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Screening Procedure for Complementation-Dependent Mutants of Vesicular Stomatitis Virus

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To isolate new types of vesicular stomatitis virus (VSV) mutants, a four-stage screen was developed which identifies and characterizes mutants capable of complementing the defect in the VSV temperature-sensitive mutant *ts*G11. Two types of mutants of VSV, Indiana serotype, have been found by using the screen; they are new temperature-sensitive mutants which are, of necessity, not in complementation group I and mutants which do not produce plaques under conditions of single infection at 31 C (the normal permissive temperature) and are, therefore, called complementation-dependent mutants. The newly isolated, temperature-sensitive mutants fall into three complementation groups, two of which are congruent with known complementation groups; the newly identified group extends to six the number of complementation groups of VSV Indiana. The nature of the complementation-dependent mutants has not been established, but one was shown to not contain a significant deletion in its nucleic acid.

Temperature-sensitive mutants of vesicular stomatitis virus (VSV), as well as host-range mutants, have been isolated in a number of laboratories (4, 6-9, 11, 13, 16). Of the five complementation groups of mutants in the Indiana serotype, complementation group I contains most of the mutants which have been isolated; it presumably corresponds to the virion-associated transcriptase which is the L (large) protein (2, 5, 12, 18). Group V appears to represent the envelope-associated G protein (21). Groups II, III, and IV have less certain associations. Mutants of the New Jersey serotype of VSV fall into six complementation groups (14).

To increase the catalog of VSV mutants, we initiated a program of isolation of conditional lethal mutants which would not necessarily depend on high temperature as the nonpermissive condition. A way was especially sought which would allow the isolation of possible amber mutants and complementing deletions (as opposed to defective, interfering particles which do not appear to complement; 15). As the procedure developed, it also automatically eliminated group I *ts* mutants and therefore generated new mutants in the other groups.

The conditional system involved identification of mutants able to grow by complementation with a known *ts* mutant at high temperature but unable to grow by themselves at high temperature. The mutants were screened by infecting cells at a high multiplicity of infection (MOI) with the *ts* mutant, co-infecting with mutagenized VSV at a low MOI, and plating the infected cells for infective centers at high temperature on a monolayer of uninfected cells. All mutants isolated this way can be called complementation dependent, but we have reserved the notation *cd* for mutants which appeared to be complementation-dependent but not temperation sensitive.

The details of the screen and some mutants which have been selected with it are described here. We caution investigators who wish to use the methods that the procedures work well as described, but that even small variations in parameters such as temperature or MOI can lead to problems.

MATERIALS AND METHODS

Cells and media. Mouse L cells were grown in continuous suspension culture at 5×10^5 to 1×10^6 cells/ml in Joklik-modified minimal essential medium (GIBCO) with added 7% fetal bovine serum. Monolayer cultures were prepared by seeding 5×10^6 L cells per 5-cm plastic tissue culture plate 1 to several hours before use. These cultures were maintained in medium supplemented with 1.8 mM $CaCl_2$, and incubations were conducted at the indicated temperatures in a humidified atmosphere of 5% CO_2. Purified virus stocks, free of interfering DI-T particles, were prepared on Chinese hamster ovary cells as

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described previously (17).

Viruses. The wild-type strain of VSV used in these experiments was the large-plaque variant of the Indiana serotype isolated and described by Wagner et al. (19). Plaque assays were carried out as previously described (19), with appropriate medium and serum for L-cell monolayers. All VSV temperature-sensitive (*ts*) mutants of the G (Glasgow) series and *ts*O45 (from the Orsay collection) were kindly supplied by C. R. Pringle.

Preparation of mutagenized virus stocks. Purified wild-type stocks of VSV were treated as described with one of the following mutagens.

(i) 5-Fluorouracil. A suspension culture of 5×10^7 L cells in 10 ml of medium with 1.8 mM $CaCl_2$ was infected with wild-type VSV at an input MOI of 0.5. After an adsorption period of 30 min at 31 C, the samples were centrifuged to remove unadsorbed virus and suspended in 10 ml of medium plus 4% serum containing 0.3 to 1.0 mg of 5-fluorouracil per ml. The infected cells were incubated in suspension for 8 h at 31 C, after which time the supernatants were collected, dialyzed against the medium, and stored at −70 C. Virus yields were 1.5×10^{-1} to 1.3×10^{-4} of those obtained in the absence of mutagen.

(ii) N-methyl-N′-nitro-N-nitrosoguanidine. Purified stock suspensions of VSV were incubated at room temperature for 15 min in 1.0 mg of nitrosoguanidine per ml by the method of Burge and Pfefferkorn (1). After dialysis against fresh medium, the samples were stored at −70 C. Survival of the virus was 2×10^{-4}.

(iii) Nitrous acid. By the procedure of Granoff (10), VSV stocks were incubated at room temperature with 1 M $NaNO_2$ in 0.25 M sodium acetate buffer (pH 4.8). At 1-min intervals, samples were removed, and the reactions were terminated by 1:100 dilutions into phosphate-buffered saline.

Complementation by infective center assay. Complementation between *ts*G11 and other *ts* mutants in individual, mixedly infected cells was assayed by plating infected cells on confluent L-cell monolayers under plaque assay conditions and then incubating the plates at the nonpermissive temperature. Suspension culture L cells were concentrated to 5×10^6 cells/ml and infected at a multiplicity of 5 PFU/cell with *ts*G11 (the helper) and 0.1 PFU/cell with the mutant to be tested (called *ts*X below). Cells infected with *ts*X alone also received 0.1 PFU/cell. After a 45-min adsorption period at the permissive temperature (31 C), the infected cells were washed twice with medium by centrifugation to remove unadsorbed virus and finally resuspended in the original volume with fresh medium. Recovery of infected cells after the centrifugation steps was approximately 50 to 60% by direct count. Appropriate dilutions were made, and 0.2-ml samples of cells were plated on confluent monolayers of uninfected L cells. Forty minutes at 31 C was allowed for attachment of the infected cells, and then an overlay consisting of 0.9% agar in medium containing 1.8 mM $CaCl_2$, nonessential amino acids, and 2.0% fetal calf serum was added. The plates were incubated for 2 days at either 31 C or 38.5 C, as appropriate, and the appearance of plaques at 38.5 C on the monolayers seeded with mixedly infected cells was interpreted as evidence for complementation in single infective centers. The results are expressed as the complementation efficiency coefficient:

Complementation efficiency coefficient for *ts*X

$$= \frac{\left[\begin{matrix} tsG11 \text{ plus } tsX \\ 38.5\text{ C titer} \end{matrix}\right] - [tsX\ 38.5\text{ C titer}]}{[tsX\ 31\text{ C titer}] - [tsX\ 38.5\text{ C titer}]}$$

The term subtracted from both numerator and denominator corrects for the presence of *ts*X revertants, generally a negligible number. L cells infected with *ts*G11 at an MOI of 5 and plated as infective centers at 38.5 C demonstrated that the complementation results were not due to *ts*G11 revertants or background growth at the nonpermissive temperature.

Examination of virus in complementation plaques (stage IV; Fig. 1). To examine the content of complementation plaques, the agar over such plaques was collected in a capillary tube, and the virus was released into 1 ml of medium as described (17). The virus was plated on L-cell monolayers which were incubated for 2 days at 31 C, and well-isolated plaques were then analyzed to determine if they contained *ts*G11, a new *ts* mutant, or something else.

For the analysis, plates containing L cells suspended in agar were used (3). For these, a 15-ml agar base layer was prepared in plastic petri dishes (15 by 100 mm); it consisted of 0.9% agar, medium, 1.8 mM $CaCl_2$, nonessential amino acids, and 2% fetal calf serum. Over this base layer was poured a suspension of 10^7 L cells in the same agar medium which was allowed to solidify. Some plates were also seeded with 4×10^5 L cells freshly infected with *ts*G11 at an MOI of 5 in the top layer in addition to the uninfected cells. The plaques to be tested were stabbed with a sterile toothpick, and the toothpick was then stabbed into identical positions on three plates of L cells in agar suspension, the last plate containing *ts*G11-infected cells. After a number of plaques had been replicated onto the three plates, one plate was incubated at 31 C, one was incubated at 38.5 C, and the *ts*G11-containing plate was incubated at 38.5 C. After 2 days, the plates were scored for growth, and the results for any one tested plaque were recorded as (+) or (−) for each of the three plates. These were incorporated into a three-place code where growth at 31 C, 38.5 C, and 38.5 C plus helper were serially represented. A result of (+ − −) was indicative of *ts*G11 and a result of (+ − +) indicated a new *ts* mutant. The (+ + +) result is discussed below.

The new *ts* mutants were harvested from the 31 C replica plates and cloned twice, and then purified stocks free of defective, interfering particles were prepared (17).

Complementation in high-MOI-infected monolayer cultures. The complementation groups of *ts* mutants were determined by a modification of the method of Cormack et al. (4). Monolayer cultures of 2.5×10^6 L cells were mixedly infected with pairs of *ts* mutants, each at an input MOI of 5. However, an MOI of 1 was used in these studies for *ts*G22 and

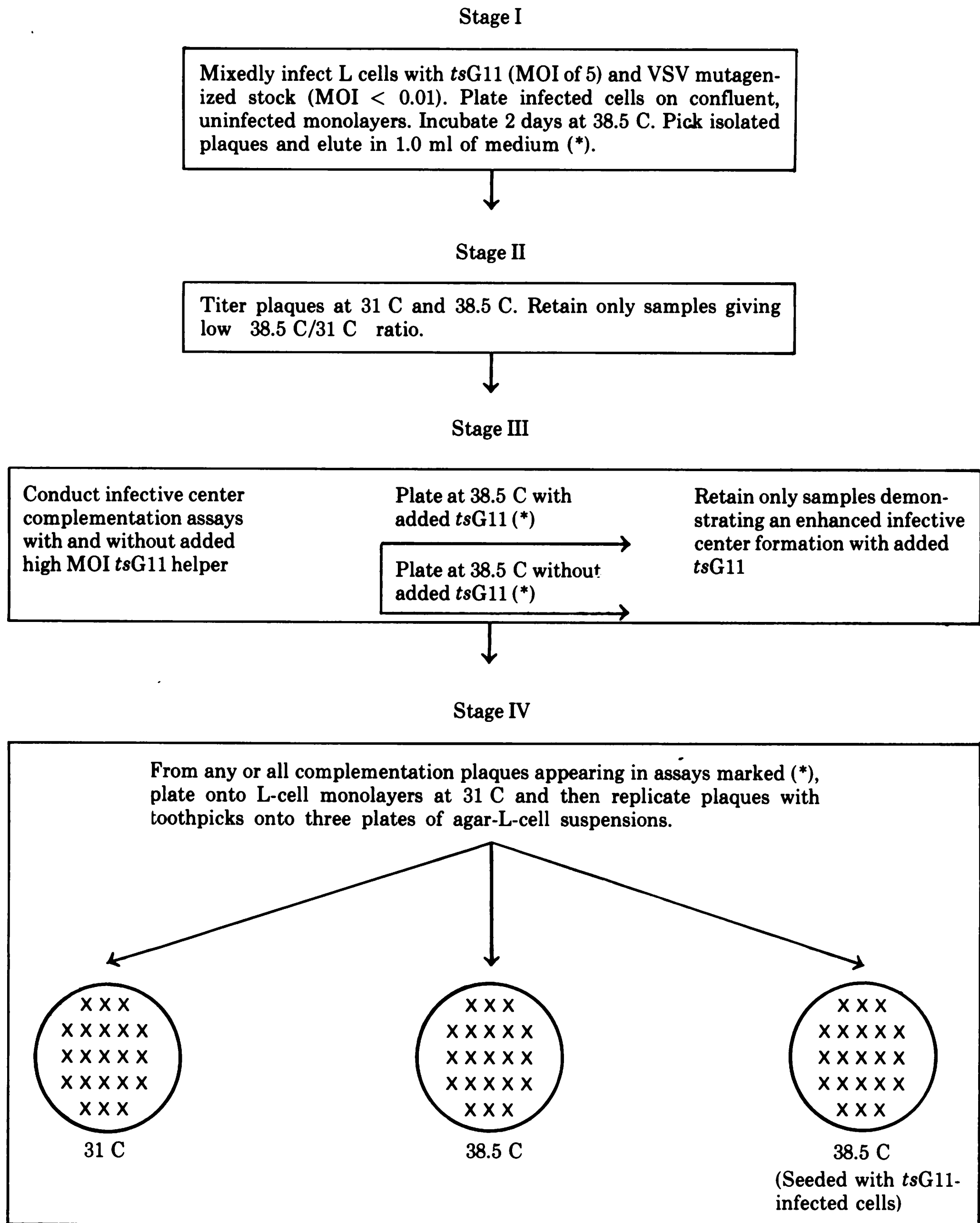

FIG. 1. *Screening procedure used to select potential complementation-dependent mutants. Asterisks (*) indicate places in the screen where 38.5 C complementation plaques were picked and examined in stage IV of the assay.*

*ts*G41 because of the high background yields obtained with these mutants. Controls for each member of a tested pair of mutants were singly infected at twice the MOI used in the mixed infection. Thirty minutes was allowed for adsorption at 39 C (39 C was used for this assay rather than 38.5 C because of the high background yield at the lower temperature). The monolayers were washed twice with 5.0 ml of medium to remove unadsorbed virus and then covered with 5.0 ml of medium with 1.8 mM $CaCl_2$, nonessential amino acids, and 2.5% fetal bovine serum. The medium was changed 90 min later to remove any virus which may have eluted from the infected cells. The supernatants were harvested 5.5 h after infection,

centrifuged to remove any floating cells, and titered at 31 C. Results are expressed as a complementation index, calculated as the ratio

$$\frac{\text{Yield } (tsA + tsB) \text{ during 39 C mixed infection}}{\text{Yield } tsA \text{ during 39 C single infection}}$$

where *ts*A and *ts*B are any pair of *ts* mutants and the yield of *ts*A at 39 C exceeds that of *ts*B.

RESULTS

Complementation between ts mutants by infective center assay. Previous studies of complementation by VSV temperature-sensitive mutants have involved mixed infections at high multiplicities in mass cultures (4, 9). Because our strategy for isolation of new mutants involved infection of all members of a population of cells by a helper mutant, infection of some cells by a single particle of a new mutant, and then determination of productive infective centers as an assay for complementation, the feasibility of this methodology was established by using known temperature-sensitive VSV mutants.

Mutant *ts*G11 acted as an efficient helper for complementation in the infective center assay with mutants from groups II, III, IV, and V. Table 1 shows the relative plating efficiency of the tested mutants at 38.5 C (nonpermissive temperature) and 31 C (permissive temperature) along with a coefficient which measures their ability to complement *ts*G11 (calculated as described in the Materials and Methods). Mutant *ts*G11 complements *ts*G22, *ts*G33, *ts*G41, and *ts*O45 efficiently enough that almost all of the particles able to form plaques at 31 C can be detected by complementation in the infective center assay. The data also confirm that *ts*G11, *ts*G13, and *ts*G114 are all in the same complementation group.

TABLE 1. *Properties of representative VSV ts mutants in L cells*

VSV mutant	Complementation group	Efficiency of plaque formation at 38.5 C[a]	tsG11 Complementation efficiency coefficient[b]
*ts*G11	I	$<10^{-6}$	
*ts*G13	I	3×10^{-5}	$<10^{-4}$
*ts*G114	I	$<10^{-6}$	$<10^{-4}$
*ts*G22	II	5×10^{-6}	0.92
*ts*G33	III	8×10^{-4}	0.97
*ts*G41	IV	2×10^{-5}	0.92
*ts*O45	V	2×10^{-5}	0.87

[a] Ratio of plaques formed at 38.5 C to those formed at 31 C.

[b] Calculated as described in Materials and Methods.

To determine if the complementation plaques arising in the infective center assay contained any recombinant progeny, the plaques were assayed for wild-type virus. More than 99% of the virus in these plaques was still temperature sensitive. A few particles able to form plaques at 38.5 C were detected, but further replating of these plaques did not yield stable recombinants. We conclude that the complementation plaques contain mainly the *ts* parent viruses plus a few aggregated particles or heteropolyploid particles in which the genomes of both parents are enclosed in a single envelope. Such particles have been previously implicated in genetic studies with VSV *ts* mutants (20).

Mutants in group I are especially well-suited as helpers in this complementation test because at high MOI they give little or no background growth in the infective center assay by themselves. Mutants from other groups were less suitable as helpers; *ts*G22 gave too high a background at 38.5 C; *ts*G41 gave only minute complementation plaques at 38.5 C, and *ts*G22 gave a complementation efficiency coefficient of 0.1 to 0.5. We therefore used *ts*G11 as the helper virus in attempts to isolate complementation dependent mutants.

Mutant screen. The procedure used to screen for complementation-dependent mutants is outlined in Fig. 1. In stage I, L cells were infected at an MOI of 5 with *ts*G11 and at an MOI of 0.01 with mutagenized wild-type VSV. These mixedly infected cells were then plated onto confluent L cell monolayers and incubated at 38.5 C (infective center assay described above). Plaques which formed would be due either to residual wild-type virus in the mutagenized stock or to complementation between a mutant and the *ts*G11 helper.

To distinguish wild-type virus from new mutants, isolated plaques from the infective center assay were eluted into 1 ml of medium. In stage II of the screen (Fig. 1), assays of 50 μliters of the plaque eluates at 31 C and 38.5 C were performed. Only samples exhibiting a very low ratio of plaques at 38.5 C to those at 31 C were retained. Such samples could have *ts*G11 plus a new *ts* mutant, *ts*G11 plus a true *cd* mutant or *ts*G11 alone. With the three mutagens used, about 5 to 10% of the stage I plaques were considered suitable for further analysis.

Those plaques retained for further analysis were then subjected to stage III of the screen to select samples containing a mutant aside from *ts*G11 which would efficiently complement

*ts*G11. For stage III, 0.1 ml of the original plaque eluates from stage I were assayed for infective center formation at 38.5 C with and without added *ts*G11. Those samples showing at least an eightfold enhancement of plaque formation at 38.5 C by *ts*G11 were selected for further analysis. About one-third of the 5-fluorouracil-treated virus which passed stage II also passed stage III (8 out of 26), 1 of 15 plaques from nitrosoguanidine-treated virus passed stage III, and 1 of 4 plaques from nitrous acid-treated virus was retained.

Stage IV of the assay was designed to distinguish new *ts* mutants from *cd* mutants and to allow isolation of the new *ts* mutants. Individual plaques arising at 38.5 C, from assays marked in Fig. 1 with an asterisk, were picked and plated at 31 C. Isolated plaques from these plates were then picked with sterile toothpicks and transferred to grid positions on three separate plates each containing L-cell suspensions in agar. One of these plates was also seeded with *ts*G11-infected cells. The *ts*G11-containing plate along with one of the other two were incubated at 38.5 C; the third plate was incubated at 31 C. The pattern of growth of virus from one tested plaque was scored as (+) or (−) on each of the three plates and recorded as a three-place code.

All plaques submitted to stage IV analysis had an excess of *ts*G11 virus because they arose due to complementation by *ts*G11. Therefore, the most frequent result at stage IV was (+ − −). In some of the plaques, a percentage of the virus gave a (+ − +) result. These were classed as non-group I, *ts* mutants because they could grow by themselves at 31 C but not at 38.5 C, and they were able to grow at 38.5 C when complemented by *ts*G11. For such mutants, the plaques on the 31 C stage IV plate were picked, the virus was recloned 2 or 3 times at 31 C, and a stock was prepared. Further analysis of such plaques is given below.

The proportion of new *ts* mutant virus in an initial 38.5 C plaque was determined from the ratio of plaques at 38.5 C with added *ts*G11 to plaques at 31 C and depended on the point on the screen from which the plaque was obtained. Stage I plaques, or stage III plaques with added *ts*G11 virus, generally contained at most 5 to 10% of the new *ts* mutants. Stage III plaques obtained without added *ts*G11 at 38.5 C often contained as much as 50% of the new *ts* mutant. Presumably, such plaques were formed either by simultaneous infection with two single viruses or, more likely, by infection with a biparental aggregate or a heteropolyploid virion. In such a case, the initial equal MOI for the two viruses might have caused the yield to be close to equal.

Stage IV results indicating true *cd* mutants will be described below.

Characterization of new temperature-sensitive mutants. The new series of mutants have been designated as M mutants to denote their origin in Massachusetts, in accordance with the recommendations for a uniform VSV *ts* mutant nomenclature (4). After the complementation analysis was completed, the *ts*M mutants were assigned numbers in a fashion similar to that of Pringle (13) where the first numeral denotes the complementation groups, whereas the last two numerals indicate the number of the mutant within the group. Table 2 presents the names of the mutants, the mutagenesis conditions which caused them, and their relative efficiency of plating at 38.5 C to that at 31 C. The diameter of plaques formed by these mutants was about equal to that of wild type after 48 h at 31 C except for *ts*M601 and *ts*M502 which required 60 to 72 h to form plaques of comparable size.

Cross-complementation of the M series of mutants was performed according to Cormack et al. (4; Table 3). They could be classified into three distinct complementation groups with *ts*5/6-03 apparently representing a double mutant.

Complementation of the M mutants with representative group I, II, III, IV, and V mutants from the Glasgow and Orsay collections was also carried out (Table 4). Mutants *ts*M301 and *ts*M302 failed to complement only *ts*G33 and therefore appeared to be in group III. Similarly, *ts*M501 and *ts*M502 could not supply

TABLE 2. *Characteristics of Massachusetts series temperature-sensitive mutants of VSV*

VSV mutant	Conditions of mutagenesis	Complementation group	Efficiency of plaque formation at 38.5 C[a]
*ts*M301	FU[b]; 0.75 mg/ml	III	4×10^{-4}
*ts*M302	FU; 0.75 mg/ml	III	9×10^{-4}
*ts*M303	NA[b]; 3 min at pH 4.8	III	8×10^{-5}
*ts*M501	FU; 0.75 mg/ml	V	5×10^{-5}
*ts*M502	FU; 1.0 mg/ml	V	5×10^{-4}
*ts*M601	FU; 0.75 mg/ml	VI	2×10^{-4}
*ts*M602	FU; 0.30 mg/ml	VI	1×10^{-3}
*ts*M5/6-03	FU; 0.30 mg/ml	V, VI	2×10^{-3}
Wild type			0.64

[a] Ratio of plaques formed at 38.5 C to those formed at 31 C.

[b] FU, 5-Fluorouracil; NA, nitrous acid.

TABLE 3. *Complementation between M series ts mutants or VSV*[a]

ts Mutant	Complementation							
	M301	M302	M303	M601	M602	M501	M502	M5/6-03
M301	(3×10^3)[b]	0.3	0.8	*19*[c]	*20*	*30*	*3*	*1.8*
M302		(4×10^2)	1.1	*140*	*60*	*50*	*4.5*	*2.3*
M303			(6×10^2)	*12*	*7.2*	*8.4*	*2.2*	*1.6*
M601				(5×10^2)	1.1	*13*	*9.2*	0.6
M602					(2×10^3)	*27*	*12*	1.2
M501						(5×10^3)	1.2	0.9
M502							(8×10^2)	0.7
M5/6-03								(4×10^4)

[a] Values in the table are expressed as a complementation index (the ratio of the mixed infection yield to the yield of the more leaky parent), and are the means from duplicate platings in one to three experiments.
[b] Values in parentheses are the virus yields obtained in single infections at 39 C in PFU/ml. Wild-type VSV gave a yield of 5×10^6 PFU/ml under these conditions.
[c] Italicized values are greater than 1.5 and represent significant complementation between the two mutants.

TABLE 4. *Complementation between VSV ts mutants derived from different wild-type strains*[a]

ts Mutant	Complementation											
	I G11	II G22	III G33	IV G41	V 045	III M301	III M302	V M501	V M502	VI M601	VI M602	V M5/6-03
G11	(6×10^4)[b]	*8.0*[c]	*34*	*6.8*	*42*	*210*	*186*	*285*	*11*	*10*	*57*	*130*
G22		(5×10^5)	*2.3*	*1.9*	*10*	*3.2*	*2.4*	*5.2*	*3.8*	*3.7*	*10*	*20*
G33			(1×10^4)	*2.1*	*3.2*	0.9	1.0	*650*	*166*	*15*	*10*	*7.0*
G41				(3×10^5)	*3.1*	*1.7*	*2.1*	*4.8*	0.3	0.4	*1.7*	*4.1*
O45					(3×10^3)	*18*	*10*	0.3	0.2	*8.9*	*4.2*	1.0

[a] Values expressed as a complementation index as described in Materials and Methods.
[b] Values in parentheses are yields obtained from singly infected controls at 39 C.
[c] Italicized values are greater than 1.5 and represent significant complementation between the two mutants.

the missing function to *ts*O45 and therefore appeared to be group V mutants. Mutant *ts*M502 did not complement *ts*G41, but the data clearly place *ts*M502 in the same group as *ts*M501 (Table 3). Mutant *ts*G41 complements poorly and *ts*M502 grows poorly even at 31 C so their lack of complementation is understandable.

It was not possible to associate mutants *ts*M601, *ts*M602, and *ts*M5/6-03 with known complementation groups. Mutant *ts*M602 appears to belong to a separate complementation group from those already characterized; we consider it group VI. The data on *ts*M601 (Table 4) suggest that it might be in group IV, but its association with *ts*M602 (Table 3) and its slow growth at 31 C make this designation unlikely. Mutant *ts*M5/6-03 complements *ts*G41 quite well; in spite of its being a double mutant, it is the best argument that group VI is different from group IV.

Properties of the cd mutants. Two plaques derived from stage I of the screen contained 5 to 20% of virus capable of complementing *ts*G11 at stage III (compared with their titers at 31 C), but no plaques giving a (+ − +) result at stage IV. These two mutants have been called *cd*M1 and *cd*M2. Table 5 presents their history along with a comparison of their behavior in stage IV assays to that of the new *ts* mutants. When over 1,000 clones derived from complementation plaques were analyzed, about 1.5% gave a (+ + +) result. Between 16 and 40% of clones derived from the *ts* mutant complementation plaques gave a (+ − +); none gave the (+ + +) result, but only 107 were analyzed.

Clones scoring as (+ + +) at stage IV for the *cd* mutants might be thought to be wild-type virus, but recloning of the virus growing without added helper at 38.5 C gave almost entirely *ts* virus with, again, an occasional (+ + +) result. Even a third serial analysis revealed no stable wild-type virus. We conclude that 1.5% of the virus in complementation plaques of the *cd* mutants are heteropolyploids or aggregates which grow at 38.5 C in the absence of helper. Aside from these rare particles, the plaques contain *ts*G11 plus 5 to 20% of a *cd* virus which

TABLE 5. *Comparison of stage IV results for cd and ts mutants*

VSV Mutant	Conditions of mutagenesis	Total No. of 31 C plaques scored[a]	No. scored plaques showing complementation[b]	Pattern of stage IV positive results[c]	
				+ − +	+ + +
*cd*M1	NG[d]; 1.0 mg/ml	628	7 (1.1)	0	7
*cd*M2	FU[d]; 0.75 mg/ml	472	8 (1.7)	0	8
*ts*M303		35	14 (40)	14	0
*ts*M501		32	5 (16)	5	0
*ts*M5/6-03		40	16 (40)	16	0

[a] Plaques arising from plating of complementation plaques (at [*] in Fig. 1) at 31 C.
[b] Percentage of total is shown in parenthesis.
[c] Positive results of stage IV assays as described in Fig. 1. The three (+) or (−) symbols represent, left to right, assays at 31, 38.5, and 38.5 C with added *ts*G11. All other tested plaques showed a (+ − −) pattern indicative of a *ts*G11 plaque.
[d] NG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; and FU, 5-fluorouracil.

does not grow by itself at 31 C or 38.5 C but will grow in cells which are co-infected by *ts*G11.

Physical properties of mutant cdM1. To produce a stock of VSV containing as high as possible a percentage of *cd*M1, a plaque originating from single infection by *ts*G11 and *cd*M1 was chosen. Such plaques are formed by aggregates or heteropolyploids and arise during stage IV assays on plates incubated at 38.5 C without added *ts*G11. One such plaque was eluted and mixed vigorously to disaggregate clumps, and its contents were analyzed by sedimentation through a sucrose gradient. Portions of the fractionated gradient were analyzed for VSV at 31 C and 38.5 C. A portion was also assayed for its ability to complement *ts*G11 in an infective center assay. Mutant *cd*M1, detected as complementing virus, cosediments with *ts*G11, detected at 31 C (Fig. 2). Mutant *cd*M1 therefore does not contain a large deletion of the VSV genome.

Virus able to grow at 38.5 C without added helper was found in two regions of the sucrose gradient (Fig. 2). Some cosedimented with the main peak of *ts*G11; it probably represents simultaneous infections of cells in the plaque assay monolayer by *ts*G11 and *cd*M1 in the peak, because dilutions of the peak fraction showed nonlinearity of plaque number at 38.5 C with dilution. Of more interest is the virus detected at 38.5 C which sedimented ahead of the main peak. In this region of the sucrose gradient, approximately equal numbers of plaques were detected with or without *ts*G11 at 38.5 C, indicating that aggregates or heteropolyploids were present (insert to Fig. 2).

DISCUSSION

We have described a new method for selecting VSV conditional lethal mutants for which the permissive condition is complementation by *ts*G11. In principle, the system could be used with other *ts* mutants as the helper in complementation, but none of the mutants we tested could be used because they were unsatisfactory in stage I of the screen.

With *ts*G11, new mutants were isolated after various mutagenesis procedures. Most of these were new *ts* mutants in complementation groups other than group I. Two mutants showed no detectable new *ts* virus and were therefore designated *cd* mutants to denote their obligate complementation dependence.

The new *ts* mutants were isolated as complementation-dependent mutants and, presumably because of the method of isolation, they all had very little virus able to grow at 38.5 C, and they all complemented well. They fell into three complementation groups when compared among themselves for complementation. One of them appeared to be in two complementation groups and was assumed to be a double mutant.

When the new *ts* mutants were compared to the existing Glasgow and Orsay mutants, two of the complementation groups of the new mutants were congruent with the standard groups. One of the complementation groups of the new mutants appeared to complement mutants from all of the existing groups; this group was therefore designated group VI, and its mutants were assigned numbers beginning with a 6. The New Jersey serotype of VSV contains six known complementation groups (14), but up to now VSV Indiana has had only five. There are only five well-established VSV Indiana proteins, so either a sixth protein exists or two of the complementation groups correspond to one protein.

The two mutants designated *cd* are clearly distinguishable from *ts* mutants in stage IV of the screen. Complementation plaques of *cd*

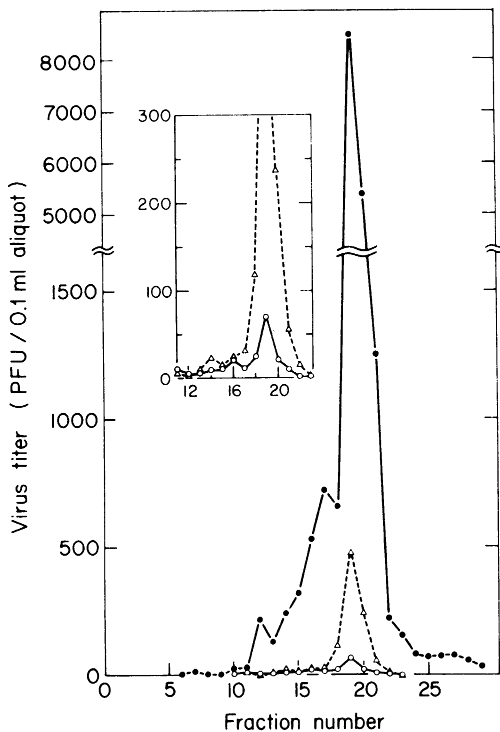

FIG. 2. *Sedimentation properties of infectious virus from a cd mutant complementation plaque. A plaque containing cdM1, which arose on a 38.5 C plate not seeded with helper in the stage IV assay (Fig. 1), was eluted in 1.0 ml of medium. This plaque eluate was layered onto 36 ml of a 5 to 40% sucrose gradient in phosphate-buffered saline and centrifuged at 21,000 rpm for 50 min at 4 C in an SW27 rotor. Sedimentation was from right to left. A hollow rod attached to tubing was inserted to the bottom of the gradient, and a Buchler polystaltic pump was used to collect 1.2-ml fractions which were titered by plaque assay at 31 C and 38.5 C. In addition, 0.1-ml samples of each gradient fraction were assayed for the presence of virus capable of complementing tsG11 by an infective center complementation assay. The insert shows fractions 11 to 23 with a more sensitive scale. Symbols: ●, 31 C titer; ○, 38.5 C titer; Δ, titer of complementing virus.*

mutants never gave rise to virus other than *ts*G11 which was capable of growth at 31 C but not at 38.5 C (Table 5). Rare progeny from complementation plaques are able to grow at 31 C and at 38.5 C in the absence of helper *ts*G11. These progeny appear to arise from mixed particles containing a *ts*G11 genome and a *cd* mutant genome; they are not wild-type virus because they never breed true.

The existence of particles with two genomes has previously been observed with VSV (20). They could be aggregates but are more likely true heteropolyploids in which two genomes are enveloped by a single membrane. Their existence is demonstrated (Fig. 2) as particles sedimenting faster than virions which score with approximately equal efficiency at 38.5 C with or without added helper.

The nature of the *cd* mutants has not been established. The one which was studied is not a large deletion mutant because it sediments at the same rate as standard VSV. In principle, *cd* mutants could be missense, nonsense, frameshift or deletion mutants. To study them in detail at this time would be difficult because the *cd* stocks contain 80 to 95% of *ts*G11. Their study will depend on the isolation of *cd* mutant stocks which contain less *ts*G11 or the development of a conditional state where they can grow by themselves. Suppressor cells growing nonsense mutants would be such a condition.

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