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Selective Isolation of Mutants of Vesicular Stomatitis Virus Defective in Production of the Viral Glycoprotein

HARVEY F. LODISH† AND ROBIN A. WEISS

Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London, WC2A 3PX, United Kingdom

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We describe a procedure that enriches for temperature-sensitive (*ts*) mutants of vesicular stomatitis virus (VSV), Indiana serotype, which are conditionally defective in the biosynthesis of the viral glycoprotein. The selection procedure depends on the rescue of pseudotypes of known *ts* VSV mutants in complementation group V (corresponding to the viral G protein) by growth at 39.5°C in cells preinfected with the avian retrovirus Rous-associated virus 1 (RAV-1). Seventeen nonleaky *ts* mutants were isolated from mutagenized stocks of VSV. Eight induced no synthesis of VSV proteins at the nonpermissive temperature and hence were not studied further. Four mutants belonged to complementation group V and resembled other *ts* (V) mutations in their thermolability, production at 39.5°C of noninfectious particles specifically deficient in VSV G protein, synthesis at 39.5°C of normal levels of viral RNA and protein, and ability to be rescued at 39.5°C by preinfection of cells by avian retroviruses. Five new *ts* mutants were, unexpectedly, in complementation group IV, the putative structural gene for the viral nucleocapsid (N) protein. At 39.5°C these mutants also induced formation of noninfectious particles relatively deficient in G protein, and production of infectious virus at 39.5°C was also enhanced by preinfection with RAV-1, although not to the same extent as in the case of the group V mutants. We believe that the primary effect of the *ts* mutation is a reduced synthesis of the nucleocapsid and thus an inhibition of synthesis of all viral proteins; apparently, the accumulation of G protein at the surface is not sufficient to envelope all the viral nucleocapsids, or the mutation in the nucleocapsid prevents proper assembly of G into virions. The selection procedure, based on pseudotype formation with glycoproteins encoded by an unrelated virus, has potential use for the isolation of new glycoprotein mutants of diverse groups of enveloped viruses.

Whereas the vesicular stomatitis virus (VSV) genome encodes the polypeptide portion of the virus glycoprotein (G), proteins required for synthesis, transmembrane insertion, glycosylation, and movement of G protein from its site of synthesis in the endoplasmic reticulum to the cell surface are contributed by the host cell. Thus the VSV G protein serves as a model for the biosynthesis of a class of cellular integral membrane glycoproteins (3, 8, 22).

Mutations in both the virus and cell genomes can be used to supplement information obtained from a biochemical dissection of glycoprotein biogenesis. A study of the growth of VSV on a number of cell lines that are defective in certain enzymes in glycosylation has provided considerable evidence concerning the mode of formation of, and functions of, the two complex oligosaccharide units attached to asparagine residues in the VSV G protein (21, 23). VSV mutations defective in glycoprotein biosynthesis are rare; indeed, Pringle characterized over 200 fluorouracil-induced temperature-sensitive (*ts*) mutations in the Indiana strain of VSV and found none in the complementation group (V) for the G gene (16-18). At least five *ts* mutations in group V are known (4, 20). In the case of the two mutants (*tsO*45, *tsM*501) that have been studied most intensively, at the nonpermissive temperature G protein is synthesized and inserted into the rough endoplasmic reticulum (rer) (10). It receives both chains of core (*N*-acetylglucosamine and mannose) sugars characteristic of the wild-type virus. The *ts* G protein, however, does not move from its site of synthesis in the rer, and no G protein is found in the cell surface (10, 11, 13). Noninfectious particles are formed that contain all of the normal VSV proteins except G (1, 19, 24).

In this paper we describe a new procedure for selective enrichment of VSV *ts* mutations specifically blocked in glycoprotein biosynthesis.

† Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.

This procedure is based on the observation that known *ts* VSV G mutations can be propagated through one cycle of growth at the nonpermissive temperature providing the cell surface contains glycoprotein of an appropriate unrelated virus, such as an avian retrovirus (30; R. A. Weiss and P. Bennett, submitted for publication). The retrovirus glycoprotein is incorporated into the budding VSV virion, forming an infectious VSV pseudotype (27, 30; Weiss and Bennett, submitted for publication).

Surprisingly, our new mutants fall into two complementation groups: IV, the presumed gene for the viral protein N, and also V. Both classes of mutants produce, at 39.5°C, particles totally or partially lacking in G protein. Other studies confirm our interpretation that one of possibly several consequences of these mutations is a defect in production of functional G protein.

MATERIALS AND METHODS

Cells and viruses. Two transformed, long-term cultures of quail cells, 16Q (15) and QT35 (14), were maintained as confluent monolayers in α medium: Dulbecco modified Eagle medium containing 10% tryptose phosphate broth, 4% heat-inactivated fetal calf serum, 1% heat-inactivated chicken serum, and 1% dimethyl sulfoxide. The 16Q clone represents a quail cell transformed by, and producing, a glycoprotein-defective Bryan high-titer strain of Rous sarcoma virus; the QT35 cell line is derived from a chemically induced sarcoma. Rous-associated virus 1 (RAV-1) is a subgroup of leukosis virus (27). 16Q or QT35 cells were infected by RAV-1 2 to 4 weeks before VSV pseudotype experiments. Leghorn chicken embryos (C/O) were supplied by Wickham Laboratories Ltd., Wickham, Hampshire, U.K. Second- or third-passage fibroblasts prepared from them were maintained in Dulbecco modified Eagle medium containing 10% tryptose phosphate broth, 1% fetal calf serum, and 1% chicken serum and were used for plaque assays of VSV irrespective of chick helper factor expression. Monkey (Vero) cells were maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum.

The Indiana serotype of VSV was used. Wild-type (*wt*) VSV and mutants *ts*G114 (I), *ts*G22 (II), *ts*G33 (III), *ts*G41 (IV), and *ts*O45 (V) (17) were kindly provided by C. Pringle (Institute of Virology, Glasgow, U.K.). Procedures for VSV plaque assays were as described previously (27), except that 0.5 ml of diluted virus (per 30-mm culture well) was used for adsorption. The medium used for all virus dilutions was complete phosphate-buffered saline (PBS) containing 1% fetal calf serum. All VSV mutants were plaque purified twice in Vero cells, and stocks were propagated at the permissive temperature, 32°C, from the least leaky clones obtained.

Virus neutralization. Hyperimmune sheep anti-VSV serum (28) was kindly provided by J. Zavada (Institute of Virology, Slovak Academy of Sciences, Bratislava, Czechoslovakia). It was always used at a dilution of 1:100, 1/10 the maximal dilution still sufficient to neutralize completely 10^9 PFU of VSV per ml in 2 h at room temperature. Neutralization tests (see Table 9) were carried out with 10^{-1} dilutions of virus; antiserum and virus were reacted at room temperature for 2 h, after which virus was diluted and plated. Complement was prepared as whole serum from pooled guinea pig blood and was stored in aliquots at −70°C. It was added to cell cultures to a concentration of 5%.

Infections with VSV. Monolayers of about 2,000 cells/mm^2 were used. Medium was removed, and 1 ml of diluted virus (in PBS) was added to each 1,500 mm^2 of culture. After a 1-h adsorption period at room temperature, the innoculum was removed and the monolayer was washed twice with PBS. Finally, 1 ml of medium was added per 300 mm^2 of monolayer, and the cultures were incubated at either 32 or 39.5°C, as indicated in the individual experiments. Before released virus or virus particles were analyzed, the medium was filtered through a 0.22-μm membrane filter (Millipore Corp.).

For labeling, the medium was standard Dulbecco modified Eagle medium containing 10% the usual concentration of methionine and 5% dialyzed fetal calf serum. The amount and type of radioisotope used is stated in each experiment.

Preparations of mutagenized virus stocks. Wild-type VSV was cloned two times at 39.5°C, and then a stock was grown, also at 39.5°C. This should reduce the proportion of spontaneous *ts* mutants.

(i) 5'-Fluorouracil and 5-azacytidine (16, 20). Cultures of Vero cells were infected with a multiplicity of 0.5 of VSV and were grown for 8 h at 32°C in the presence of 0.30 mg of 5-fluorouracil (FU) or 0.004 mg of 5-azacytidine per ml. The efficiency of plating of either stock at 39.5°C, relative to 32°C, was 0.6 (*wt* VSV being 0.8), indicating that about 25% of the infectious VSV were *ts*.

(ii) Nitrous acid (20). Before mutagenesis, a stock of *wt* VSV was concentrated 50-fold by centrifugation. It was diluted into a solution containing 0.25 M sodium acetate, pH 4.8, and 1 M sodium nitrite. After incubation for 30 s at room temperature, the reaction was terminated by a 100-fold dilution into PBS containing 5% fetal calf serum. Again, about 30% of the resultant VSV were *ts*.

Selection of *ts* mutants defective in G-protein synthesis. A culture of RAV-1-infected 16Q cells was infected with a multiplicity of 0.1 of a mutagenized population of VSV and was incubated at 39.5°C. After 1 h, anti-VSV serum and complement (see above) were added, and incubation continued at 39.5°C for 18 h. The resultant supernatant virus was plaqued on monolayers of C/O cells at 32°C (see Table 1). For selection of mutants, a dilution of virus was chosen so that there were from one to three plaques on each 30-mm-diameter monolayer. Individual plaques were picked into 1 ml of medium. Five microliters of this was assayed for plaques on monolayers of Vero cells at 32 and 39.5°C. Candidate *ts* mutants were cloned twice on Vero cells at 32°C before large stocks were grown, also at 32°C.

Thermal inactivation. Ten-fold dilutions of virus stocks in PBS containing 10% fetal calf serum were placed in sealed vials and immersed in a 45°C water bath for 60 min. Infectious VSV was assayed by pla-

quing dilutions on monolayers of Vero cells at 32°C.

Glycoprotein-defective VSV particles. Three milliliters of filtered medium from [^{35}S]methionine-labeled, infected cells was layered atop 2.5 ml of 20% (wt/wt) sucrose in PBS. Centrifugation was for 45 min at 45,000 rpm and 4°C in a Beckman SW50.1 rotor. The pellet was rinsed once with PBS and resuspended overnight in 400 μl of PBS. A portion of the pellet was taken for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. A portion was layered onto a 5.2-ml linear 20 to 50% (wt/wt) sucrose gradient, made up in PBS, and centrifuged to equilibrium—16 h at 25,000 rpm and 4°C in the SW50.1 rotor. Fractions were collected by pumping from the bottom of the tube; aliquots were taken for determination of refractive index and for ^{35}S radioactivity precipitable in 10% trichloroacetic acid. Precipitates were collected on fiberglass filters and counted in a toluene-2,5-diphenyloxazole solution in a Packard scintillation counter.

Polyacrylamide gel electrophoresis. To analyze labeled intracellular protein, monolayers of infected cells were washed once with PBS and then disrupted directly by addition of electrophoresis dissociation buffer. Virus pellets or samples from sucrose gradients were disrupted by twofold dilution into 2×-concentrated dissociation buffer. Proteins were separated by electrophoresis through slab polyacrylamide gels containing SDS as described by Laemmli (12). After electrophoresis, the gels were fixed in 10% trichloroacetic acid at 4°C for 1 h, washed in water for 2 h, and then dried under vacuum. Autoradiography used Kodak X-Omat H1 X-ray film, and the films were scanned with a Joyce-Loebl microdensitometer using a wedge such that full-scale pen deflection was 1.0 optical density unit. Under these conditions, blackening of the film and the area under the peak was proportional to the amount of radioactivity in the protein band.

Virus RNA synthesis. Actinomycin D (5 μg/ml) was added to the cells immediately after infection, and [^{3}H]uridine (100 μCi/ml, 1,000 mCi/mmol) was added at 3 h. After an additional 3 h of incubation, the medium was removed, and the cells were washed once with PBS. The cells were disrupted by addition of 1 ml of PBS containing 1% Triton X-100, 1% deoxycholate, and 0.1% diethylpyrocarbonate; this extracts all VSV-specific RNA but leaves the nuclei still attached to the culture dish. A 100-μl sample was precipitated with trichloroacetic acid, and the precipitate was collected and counted as above.

RESULTS

Scheme for selection of mutants *ts* for G-protein synthesis. When avian cells that are not expressing any retrovirus glycoprotein are infected at 39.5°C by *ts*O45 (V), noninfectious virus particles lacking the viral G protein are formed (1, 24). These will not form plaques on any avian or mammalian cell tested. However, *ts*O45 infection of a cell previously infected by a leukosis virus, such as RAV-1, does result in production of infectious particles. These are *ts*O45(RAV-1) pseudotypes, containing the surface glycoprotein of the RAV-1 virus, and are able to infect only those cells bearing receptors for this leukosis virus. The infectivity of these particles is totally resistant to anti-VSV serum, even in the presence of complement, so they contain no detectable VSV G protein (30; Weiss and Bennett, submitted). Thus, one can propagate through one cycle at the nonpermissive temperature a VSV *ts* mutant blocked in G-protein synthesis. [*ts*O45 will not plaque on RAV-1 cells at 39.5°C; the *ts*O45(RAV-1) pseudotypes produced during the first cycle of infection cannot reinfect the RAV-1 cells because the receptors are blocked by RAV-1 glycoproteins (26).] We were able to use this as the basis of a procedure to enrich for *tsG* mutants.

A line of quail cells, 16Q, previously infected with RAV-1, is infected with 0.1 PFU of a mutagenized stock of VSV per cell and incubated at 39.5°C. Cells that express on their surface VSV G protein are lysed with anti-VSV serum and complement. The only infectious VSV particles which should result are VSV(RAV-1) pseudotypes derived from cells that are expressing no VSV G protein on their surface. These are detected as PFU on sensitive (C/O) chick cells at 32°C. Table 1 shows that when cells preinfected with RAV-1 and infected with *wt* VSV were incubated at 39.5°C in medium containing anti-VSV serum and complement, the yield of infectious virus was reduced by a factor of 2×10^4. The small number of infectious particles that remained were VSV(RAV-1) pseudotypes; they plaqued only on cells bearing receptors for RAV-1 (data not shown; Weiss and Bennett, submitted). As expected for *wt* VSV, those particles plaqued with normal efficiency at 39.5°C relative to 32°C; presumably they arose from cells that, for unknown reasons, were expressing little VSV G protein.

At 39.5°C in the presence of anti-VSV serum and complement, the yield of infectious particles after infection by *ts*O45 was 10 to 20 times greater than that by *wt* VSV (Table 1). Cells infected by *ts*O45 at 39.5°C should not be (Weiss and Bennett, submitted), and apparently were not, affected by anti-VSV serum and complement. These infectious particles were *ts*O45-(RAV-1) pseudotypes; they plaqued only at 32°C and only on cells bearing receptors to RAV-1 (data not shown). Significantly, no infectious particles were produced at 39.5°C in the presence of anti-VSV serum and complement after infection by *ts* mutants in the other four complementation groups (Table 1). Those infected cells were unable to form either the viral RNA or structural proteins required for production either of infectious virus or of RAV-1 pseudotypes.

Thus, growth of a mutagenized population of

TABLE 1. *Growth of VSV ts mutants at 39.5°C in the presence of anti-VSV serum and complement*[a]

Virus	PFU/ml	
	16Q	16Q preinfected with RAV-1
Expt 1		
*tsG*114 (I)	<20	<20
*tsG*22 (II)	<20	<20
*tsG*33 (III)	<20	<20
*tsG*41 (IV)	<20	<20
*tsO*45 (V)	<20	8.4×10^3
Wild type	<20	4.6×10^2
Expt 2		
Wild type, no antiserum or complement	1.6×10^7	1.1×10^7
Wild type, antiserum only	<20	6.6×10^3
Wild type, complement	ND[b]	1.1×10^7
Wild type, both antiserum and complement	ND	6.0×10^2

[a] Monolayers containing 5×10^6 16Q or 16Q RAV-1-preinfected cells were infected with 6×10^5 PFU of wild-type VSV or the indicated mutants. After 1 h of adsorption, the monolayers were washed twice to remove unadsorbed virus. Ten milliliters of medium was added, and the cultures were plated at 39.5°C. After 1 h, antiserum and guinea pig complement (see text) were added as indicated. The yield of virus after a total of 18 h of incubation was assayed by plaquing at 32°C on sensitive C/O cells.

[b] ND, Not done.

VSV at 39.5°C in an RAV-1-infected cell in the presence of anti-VSV serum and complement should select against *ts* mutants in any complementation group other than V. The proportion of group V mutants relative to wt VSV should be enriched 10-fold. The actual proportion of *ts* (V) mutants emerging from this selection will, of course, depend on the (unknown) fraction of *ts* (V) and *ts* mutants in other genes present in the original mutagenized population.

Isolation of *ts* mutants. To reduce the frequency of spontaneous *ts* mutants, VSV was first cloned two times at 39.5°C. As indicated in Materials and Methods, stocks were mutagenized with FU, 5-azacytidine, or HNO_2 to an extent that about 25% of the infectious virus was temperature sensitive. These stocks were used to infect, at 39.5°C, RAV-1-producing 16Q cells; as in Table 1, the cultures were treated with anti-VSV serum and complement. The resulting stocks were plated on C/O cells at 32°C. About 900 individual plaques were picked and tested for ability to plaque at 32 and 39.5°C. Irrespective of the mutagen, about 1 in 35 plaques was *ts*, plaquing only at 32°C.

The first 26 cloned *ts* mutants were selected for further study. Of these, eight were discarded since the yield of infectious virus at 39.5°C was over 2% that of wild type, making genetic analysis impossible. One clone was lost. To characterize the remaining 17 mutants in a preliminary fashion, cultures were labeled with [^{35}S]methionine from 3 to 5 h after infection, and synthesis of virus-specific proteins was measured after SDS-polyacrylamide gel analysis of radioactive proteins (see Fig. 1). Eight mutants induced, at 39.5°C, the synthesis of less than 10% the amount of virus protein as did *wt* VSV. These were not studied further since they obviously contained lesions affecting some other aspect of virus biogenesis in addition to any possible direct effect on G protein synthesis.

Essential characteristics of the nine remaining new *ts* mutants are presented in Table 2. Each mutant was cloned twice on Vero cells at 32°C before a large stock was grown. Note that stocks of all mutants contained between 8×10^{-5} and 2×10^{-4} *wt* revertant plaques, as indeed did our cloned stocks of *tsO*45 (V) and *tsG*33 (III). We believe that this rate of reversion to a temperature-resistant virus is that characteristic of a single *ts* mutation, although we do not know whether the revertant plaques (formed at 39.5°C) represent a change at the original mutated site.

Complementation studies. To our surprise, the new *ts* mutants fell into two clear complementation groups, IV and V. Two different types of complementation tests were used.

In the first (complementation by plaque formation), we took advantage of our finding that monolayers of Vero cells, infected at 39.5°C by high multiplicities of *ts* mutants *tsG*114 (I), *tsG*22 (II), and *tsG*41 (IV), did not lyse, even after 3 days. If such infected monolayers were also infected with small numbers of another *ts* VSV mutant and then overlaid with agar, plaques could form by continuous complementation. Indeed, *tsO*45 (V), *tsG*33 (III), and all of our new *ts* VSV mutants formed, at 39.5°C, plaques at rather high efficiency on monolayers preinfected with *tsG*114 (I) or *tsG*22 (II) (Table 3). This indicates that none of these mutants could have a defect in genes of complementation groups I or II. Some of the new mutants (*tsL*511–514) also plaqued on lawns seeded with *ts*41 (IV); clearly these were not group IV mutants either. However, five new mutants, *tsL*411–415, did not form plaques at 39.5°C on monolayers seeded with *tsG*41. Although this result could not itself establish that these mutants fell into complementation group IV, the following complementation tests showed that this was, indeed, the case.

TABLE 2. *Characteristics of new VSV ts mutants*[a]

Virus	Mutagen[b]	Reversion frequency (PFU at 39.5/32°C)	PFU produced at 39.5/32°C	Yield of virus-like particles produced at 39.5°C, relative to *wt*	Infectivity of particles produced at 39.5°C, relative to *wt*	G/(N + M) in particles produced at 39.5°C
Wild type		0.80	2.4	1.00	1.00	0.27 (1.00)
*L*411 (IV)	FU	3.3×10^{-5}	1.6×10^{-3}	5.0×10^{-3}	5.0×10^{-2}	0.18 (0.68)
*L*412 (IV)	AC	1.0×10^{-4}	1.2×10^{-2}	2.0×10^{-2}	11.8×10^{-1}	0.14 (0.58)
*L*413 (IV)	AC	4.4×10^{-5}	2.2×10^{-2}	1.7×10^{-1}	2.1×10^{-1}	0.18 (0.75)
*L*414 (IV)	HNO_2	1.5×10^{-5}	1.9×10^{-3}	4.5×10^{-3}	2.0×10^{-1}	0.10 (0.41)
*L*415 (IV)	FU	6.6×10^{-5}	1.4×10^{-3}	3.0×10^{-2}	1.0×10^{-1}	0.09 (0.38)
*L*511 (V)	FU	7.5×10^{-6}	4.2×10^{-5}	4.0×10^{-3}	4.3×10^{-3}	0.007 (0.03)
*L*512 (V)	FU	8.7×10^{-5}	2.0×10^{-2}	1.7×10^{-2}	1.1×10^{-1}	0.028 (0.11)
*L*513 (V)	FU	1.8×10^{-4}	6.0×10^{-5}	2.9×10^{-2}	3.3×10^{-2}	0.005 (0.020)
*L*514 (V)	HNO_2	7.6×10^{-5}	1.0×10^{-4}	2.8×10^{-2}	3.3×10^{-2}	0.006 (0.025)
*tsG*41 (IV)		$<10^{-8}$	ND[c]	$<1 \times 10^{-3}$		
*tsO*45 (V)		2.6×10^{-5}	4.0×10^{-4}	2.5×10^{-2}	1.7×10^{-3}	0.005 (0.020)

[a] To obtain the data in the last four columns, monolayers of 2×10^6 QT35 cells in 35-mm dishes were infected by 2 PFU/cell. After the 1-h period at room temperature allowed for adsorption, the infecting virus was removed, and 2 ml of medium containing reduced methionine was added. Cultures were incubated at 32 or 39.5°C for an additional 8 h; at 3 h, 300 μCi of [^{35}S]methionine was added to the 39.5°C cultures and 30 μCi was added to the 32°C ones. Infectious virus produced was assayed in Vero cells (at 32°C). Two milliliters of filtered cell supernatant was centrifuged through a 3.5-ml cushion of 20% (wt/wt) sucrose in PBS, and 1/10 of the resultant virus pellet was analyzed by SDS-polyacrylamide gel electrophoresis (see text). The relative amount of virus particles was calculated from the total amount of radioactivity in the N and M polypeptides (see Fig. 2); similarly, the ratio of radioactivity in G to the sum of N and M was determined from the areas under the peaks of these scans.

[b] FU, 5′-Fluorouracil; AC, 5′-azacytidine.

[c] ND, Not done.

TABLE 3. *Efficiency of plating VSV ts mutants at 39.5°C on monolayers of cells containing helper VSV relative to 32°C*[a]

Virus	No. of helper	Plus *tsG*114 (I)	Plus *tsG*22 (II)	Plus *tsG*41 (IV)
*tsL*411 (IV)	$<10^{-3}$	0.33	0.68	$<10^{-3}$
*tsL*412 (IV)	$<10^{-3}$	0.15	0.34	$<10^{-3}$
*tsL*413 (IV)	$<10^{-3}$	0.55	0.64	$<10^{-3}$
*tsL*414 (IV)	$<10^{-3}$	0.51	1.03	$<10^{-3}$
*tsL*415 (IV)	$<10^{-3}$	0.66	0.91	$<10^{-3}$
*tsL*511 (V)	$<10^{-3}$	0.33	0.08	0.11
*tsL*512 (V)	10^{-2} t[b]	0.52	0.66	0.13
*tsL*513 (V)	$<10^{-3}$	0.68	1.46	0.23
*tsL*514 (V)	$<10^{-3}$	0.21t	0.91	0.11
*tsO*45 (V)	$<10^{-3}$	0.16t	0.34	0.048
*tsG*33 (III)	$<10^{-3}$	0.25	0.25	0.013

[a] Three 10-fold serial dilutions of each virus stock (containing between 10 and 10,000 PFU in 0.3 ml) were added to monolayers of 2×10^6 Vero cells in 35-mm culture dishes. Cells also received 0.2 ml of either (i) saline or (ii) saline containing 2×10^6 PFU of *tsG*114 (I), 4×10^6 PFU of *tsG*22 (II), or 10^6 PFU of *tsG*41 (IV). After a 1-h period at room temperature, the virus inoculum was removed and the plates were overlaid with 2.5 ml of agar. Sets of control plates (without helper) were incubated at 32°C (to determine the total PFU) and at 39.5°C; plates with helper were incubated at 39.5°C. All plates with helper, but no tester VSV, yielded no plaques at 39.5°C.

[b] t, Tiny plaques.

In these standard complementation tests, monolayers of QT35 cells were mixedly infected, at 39.5°C, by 5 PFU of each of two *ts* mutants per cell. The yield of infectious virus that resulted was determined by plaquing on Vero cells at 32°C (Table 4). Positive complementation is said to occur when the ratio (complementation index) of the yield from the doubly infected culture to the sum of the yields from the two singly infected cultures is greater than 2. Mutants *tsL*411, -*L*412, -*L*413, -*L*414 (Table 4), and -*L*415 (not shown) clearly complemented mutants in groups I, II, III, and V, but not *tsG*41 (IV). Thus, these mutants indeed were in group IV. [We do not know why the yield from cultures infected by two group IV mutants, *tsG*41 (IV) and *tsL*412 (IV), was less than in cultures infected by only one.]

Results with the other four new mutants, *tsL*511, -*L*512, -*L*513 (Table 4), and -*L*514 (not shown), appear less clear-cut. They complemented mutants in groups I, II, and III, but not *tsG*41 (IV) or *tsO*45 (V). However, two pieces of evidence show unambiguously that these new mutants could complement group IV mutants and therefore must be only in complementation group V. First, these four formed plaques at 39.5°C on monolayers seeded with *tsG*41 (IV) (Table 3). Second, and more important, all of

TABLE 4. *Complementation between VSV ts mutants*[a]

Virus	Yield from singly infected cells at 39.5°C (PFU/ml)	Complementation index with:				
		*tsG*114 (I)	*tsG*22 (II)	*tsG*33 (III)	*tsG*41 (IV)	*tsO*45 (V)
*tsG*114 (I)	1.8×10^3					
*tsG*22 (II)	8.6×10^3	17				
*tsG*33 (III)	6.6×10^4	12	1,500			
*tsG*41 (IV)	5.8×10^3	2.4	6	2.2		
*tsO*45 (V)	5.4×10^3	11	1,500	25	1.6	
*tsL*411 (IV)	8.2×10^4	47	180	88	0.17	390
*tsL*412 (IV)	8.2×10^5	1.8	9	4	0.002	3.2
*tsL*413 (IV)	1.4×10^6	2.2	10	5	0.01	4.3
*tsL*414 (IV)	4.3×10^5	8.0	30	41	0.03	68
*tsL*511 (V)	9.2×10^4	2.7	380	29	1.40	0.07
*tsL*512 (V)	2.1×10^5	7.6	11	7.1	0.20	0.01
*tsL*513 (V)	2.2×10^4	6.8	650	136	1.80	0.19

[a] Monolayers of 2×10^6 QT35 cells were infected with a multiplicity of five of one, or each of two, VSV mutants and were incubated for 8 h at 39.5°C. The yield of infectious virus was determined by plaquing on Vero cells. The complementation index is the ratio of yields from the doubly infected culture to the sum of the yields from the two singly infected cultures. The yield from each of the singly infected cells is given in column 2.

these new *ts* mutants complemented all of the newly isolated group IV mutants. Results of some of these complementation tests are illustrated in Table 5. Note also that the prototype *tsO*45 (V) did not complement *tsG*41 (IV) but did complement all of the new group IV mutants (Table 4).

Characterization of new *ts* mutants: RNA and protein synthesis. To measure the rate of virus RNA synthesis, infected cells were treated with actinomycin D to block residual cellular RNA production and then were labeled with [^{3}H]uridine from 3 to 6 h. Cells infected by *wt* VSV at either 32 or 39.5°C incorporated 8 to 10 times the amount of ^{3}H radioactivity into RNA as did uninfected cells (Table 6). Cells infected with *tsO*45 (V) or any of the new group V mutants synthesized essentially normal amounts of viral RNA, either at 32 or at 39.5°C. By contrast, none of the cells infected at 39.5°C by group IV mutants accumulated more than background levels of [^{3}H]RNA; RNA synthesis was, except for *tsG*41 (IV) and *ts*411 (IV), more or less normal at 32°C (Table 6).

The absence of detectable RNA synthesis at 39.5°C by cells infected by group IV mutants was surprising since these cells did synthesize large amounts of viral proteins (Fig. 1, Table 7). In particular, cells infected by *tsL*413 (IV) synthesized at 39.5°C 30 to 50% the amount of virus polypeptides G, N, and M as did cells infected by *wt* VSV or *ts* (V) mutants. L and NS proteins could not be resolved from background cell proteins. In suspension CHO cells infected by VSV, about 80% of the virus-specific RNA synthesized at 4 h is mRNA (7, 11), but we do not know whether this is true for our Vero cells. It is possible that cells infected by *wt* VSV synthesize VSV mRNA in considerable excess over that required for virus protein synthesis. Cells infected by the group IV mutants could be making less than 10% the normal amount of viral mRNA, but this would still be sufficient for synthesis of considerable numbers of viral polypeptides.

Thermolability. The infectivity of stocks (grown and assayed at 32°C) of all the new group V mutants, as well as of *tsO*45 (V) (9, 30; Weiss

TABLE 5. *Complementation between some new ts VSV mutants*[a]

Virus	Yield from singly infected cells at 39.5°C (PFU/ml)	Complementation index with:			
		*tsL*412 (IV)	*tsL*414 (IV)	*tsL*415 (IV)	*tsL*511 (V)
*tsL*411 (IV)	7.4×10^4	0.6	1.2	ND[b]	ND
*tsL*412 (IV)	3.8×10^3		1.3	0.6	503
*tsL*413 (IV)	6.2×10^5	1.4	ND	ND	ND
*tsL*414 (IV)	6.8×10^4	1.3		0.9	95
*tsL*415 (IV)	6.5×10^4	0.6	0.9		120
*tsL*511 (V)	2.6×10^4	503	95	120	
*tsL*513 (V)	1.4×10^3	770	27	88	1.2
*tsO*45	2.6×10^3	812	46	39	0.8

[a] Complementation was done as described in the legend to Table 4, except that Vero cells were used throughout.

[b] ND, Not done.

TABLE 6. *Virus-specific RNA synthesis in infected cells*[a]

Virus	[^{3}H]uridine (cpm × 10^{-3})	
	32°C	39.5°C
None	8.5	8.6
Wild type	86.4	60.1
*tsG*114 (I)	64.0	7.1
*tsG*22 (II)	67.6	8.2
*tsG*33 (III)	73.0	44.8
*tsG*41 (IV)	14.6	6.8
*tsO*45 (V)	69.7	49.2
*tsL*411 (IV)	14.8	7.4
*tsL*412 (IV)	116.4	8.9
*tsL*413 (IV)	41.8	7.6
*tsL*414 (IV)	82.1	6.9
*tsL*415 (IV)	77.0	8.8
*tsL*511 (V)	75.0	23.0
*tsL*512 (V)	49.0	38.7
*tsL*513 (V)	70.7	85.0
*tsL*514 (V)	69.8	44.8

[a] Monolayers of Vero cells were infected, treated with actinomycin D, and labeled with [^{3}H]uridine from 3 to 6 h, as detailed in Materials and Methods. Shown is the amount of acid-precipitable radioactivity per 2 × 10^5 cells. Background radioactivity from uninfected cells has not been subtracted. Although not shown here, other infected cells were harvested at 4.5 h, i.e., after 1.5 h of labeling; in all cases, the amount of acid-precipitable radioactivity was about half that indicated here.

and Bennett, submitted), was reduced over 100-fold by heating for 60 min at 45°C (Table 8). All of the new *ts* (IV) mutants, like *tsG*41 (IV) and *wt* VSV, were resistant to heat inactivation.

Production of noninfectious particles. Cells infected at 39.5°C by any of these new mutants produced noninfectious particles that were deficient in viral glycoprotein. This could be seen by labeling the cells with [^{35}S]methionine from 3 to 8 h after infection. The yield of infectious particles could be determined by plaque assay of the filtered medium. A portion of the filtered medium was centrifuged through a cushion of 20% sucrose, and the material in the pellet (particulate fraction) was analyzed by SDS-gel analysis. Both the nature and amount of virus polypeptide could be determined from scans of these gels (Fig. 2, Table 2).

Infection at 39.5°C by three of the four new group V mutants, like *tsO*45 (24), produced par-

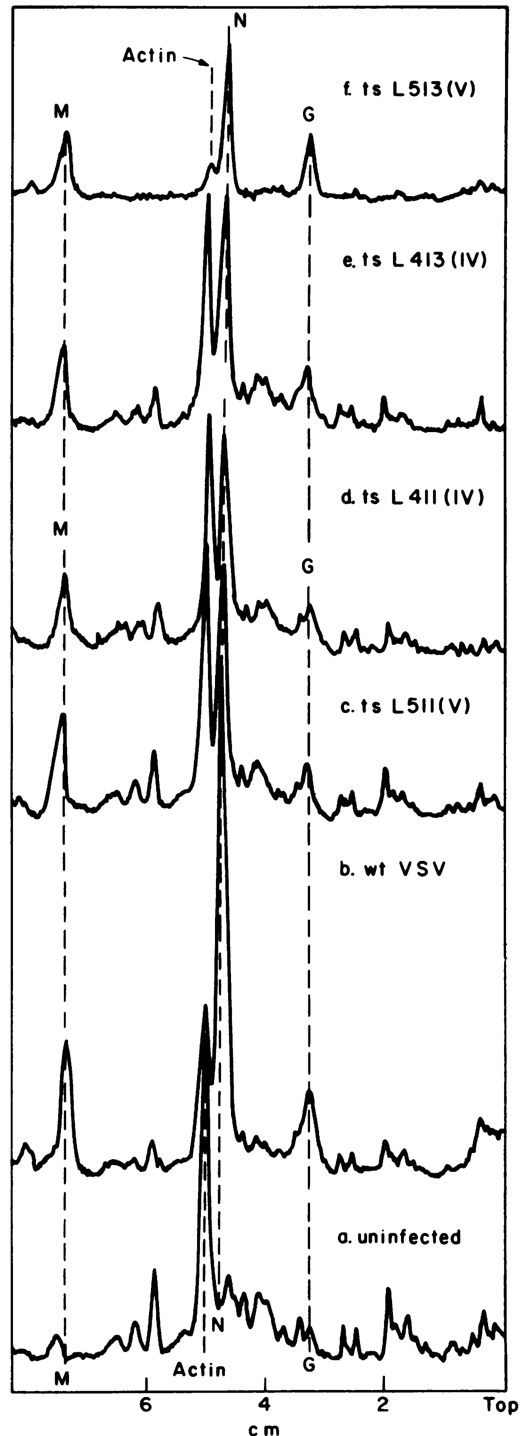

FIG. 1. *Synthesis of VSV proteins in infected cells at 39.5°C. Monolayers of Vero cells were infected with VSV mutants and labeled with [^{35}S]methionine, as detailed in the legend to Table 7. Shown here are scans of autoradiograms of SDS-slab gel separations of labeled VSV proteins; the amount of individual VSV proteins made is proportional to the area under the respective peaks and is tabulated in Table 7. The peak at 5 cm comigrates with cellular actin, but its identity as actin has not been proven. VSV NS and L proteins are not resolved here from residual host polypeptides.*

TABLE 7. *Synthesis of virus polypeptides at 39.5°C*[a]

Mutant	Polypeptide		
	G	N	M
Wild type	19 (1.00)	154 (1.00)	43 (1.00)
*tsL*411 (IV)	6 (0.31)	53 (0.34)	25 (0.58)
*tsL*412 (IV)	4 (0.21)	39 (0.25)	17 (0.38)
*tsL*413 (IV)	12 (0.63)	61 (0.40)	27 (0.63)
*tsL*414 (IV)	7 (0.36)	42 (0.27)	19 (0.44)
*tsL*511 (V)	9 (0.47)	69 (0.45)	34 (0.79)
*tsL*512 (V)	53 (2.8)	323 (2.1)	86 (2.0)
*tsL*513 (V)	10 (0.53)	36 (0.23)	19 (0.44)

[a] Monolayers of 2×10^6 Vero cells were infected with a multiplicity of five of the indicated viruses; actinomycin D (5 μg/ml) was added, and the cells were incubated at 39.5°C for 4 h. Fifty microcuries of [^{35}S]methionine (100,000 mCi/mmol) was added, and incubation continued for 35 min. As detailed in the text, the cells were dissolved in gel dissociation buffer, and 1/10 was taken for SDS-gel electrophoresis. Film was exposed to the dried gel for 17 h, and the autoradiogram was scanned (see Fig. 1). Data show the area under the VSU G, N, and M regions, in arbitrary units, that is proportional to the amount of radioactivity in these polypeptides. Backgrounds from uninfected cells (G, 9 units; N, 17 units; M, 0 units) have been subtracted. Indicated in brackets is the fraction of material found after infection by wild-type virus.

TABLE 8. *Thermal inactivation of VSV mutants*[a]

Virus	Log_{10} of fraction surviving for 60 min at 45°C
Wild type	−0.12
*tsG*41 (V)	−0.11
*tsO*45 (V)	−3.5
*tsL*411 (IV)	−0.12
*tsL*412 (IV)	−0.17
*tsL*413 (IV)	−0.03
*tsL*414 (IV)	−0.06
*tsL*415 (IV)	−0.11
*tsL*511	−2.0
*tsL*512	−3.2
*tsL*513	−4.9
*tsL*514	−4.3

[a] VSV stocks were grown at 32°C in Vero cells, and 10^{-1} dilutions were inactivated as described in Materials and Methods and plaque assayed at 32°C on Vero cells.

ticles containing N and M proteins in the normal proportion but containing less than 3% the normal level of G protein. The other, *ts*512 (V), produced particles containing about 10% the normal proportion of G protein (Fig. 2, Table 2). Irrespective of the mutant, the infectivity of these glycoprotein-deficient particles (relative to *wt* particles) was less than 10% (Table 2).

Infection at 39.5°C by three of the four new group V mutants, like *tsO*45 (24), produced particles containing N and M proteins in the normal proportion but containing less than 3% the normal level of G protein. The other, *ts*512 (V), produced particles containing about 10% the normal proportion of G protein (Fig. 2, Table 2). Irrespective of the mutant, the infectivity of these glycoprotein-deficient particles (relative to *wt* particles) was less than 10% (Table 2).

Infection at 39.5°C by any of the five new group IV mutants resulted in production of particles with a specific infectivity of 5 to 20% that of wild type. Depending on the mutant, these contained between 35 and 75% of the normal proportion of G protein (Fig. 2, Table 2).

Most of these noninfectious particles had a density in sucrose indistinguishable from that of *wt* particles. Examples of this are *tsL*511 (V) (Fig. 3b) and *tsL*411 (IV), *tsL*413 (IV), and *tsL*414 (IV) (not shown). Particles produced by several of the new mutants, like those produced by *tsO*45 (V) (24), had a lighter density than normal *wt* particles. Examples of this class include *tsL*412 (IV) (Fig. 3a) and *tsL*512 (V) and *tsL*513 (V) (Fig. 3d and c, respectively).

Formation of RAV-1 pseudotypes. The selection of these mutants depended both on the absence, at 39.5°C, of appreciable VSV G protein at the cell surface and on the ability of the mutants to be rescued by RAV-1. Because several new mutants fell into complementation group IV, not V as expected, it was necessary to investigate in more detail the effects of RAV-1 preinfection on the replication of these mutants at 39.5°C.

Infection of QT35 RAV-1-producing cells at 39.5°C by *tsL*511 (V) or *tsL*513 (V) resulted in production of 25 times as many infectious particles as did infection of QT35. This is similar to the 80-fold stimulation of replication of *tsO*45 (V). Essentially all of the infectivity of these lysates was resistant to anti-VSV serum, suggesting that these were pure RAV-1 pseudotypes and that the particles contained little, if any, VSV G protein (Weiss and Bennett, submitted). Further evidence that these were pure RAV-1 pseudotypes was the observation (data not shown) that they plaqued only on cells, such as C/O, that bear receptors for RAV-1. Replication of *tsL*512 (V) at 39.5°C was enhanced only 10-fold by preinfection by RAV-1, and only 30% of the particles were resistant to anti-VSV serum. This mutant seemed a bit leakier than the others (see Table 2), and apparently some VSV G protein was incorporated into the RAV-1 pseudotypes as it was into noninfectious particles (Table 2).

Replication of the four group IV mutants tested was also stimulated by preinfection by RAV-1, but only three- to fivefold (Table 9). In

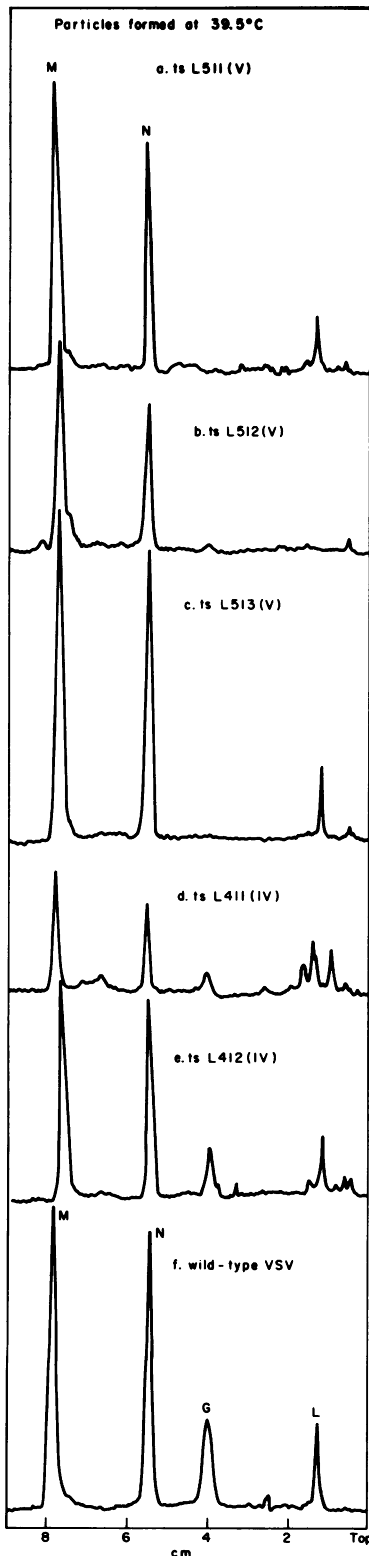

FIG. 2. *Polypeptide composition of defective viral particles produced at 39.5°C. As detailed in the legend to Table 2, QT35 cells were infected with VSV mutants, incubated at 39.5°C, and labeled with [^{35}S]methionine from 3 to 8 h. A portion of the particulate fraction from the filtered extracellular medium was analyzed by SDS-gel electrophoresis. Scans of the radioautograms of the dried gels are shown. Films were exposed for between 4 and 72 h, depending on the amount of radioactivity in the particulate fraction, such that the blackening of the film was in the 1.0 optical density unit linear range of the microdensitometer. Tabulated in Table 2 is the amount of radioactivity in viral polypeptides, as well as the relative amount of G protein in these particulates.*

contrast to the group V pseudotypes, only 1 to 3% of the infectious particles produced in the RAV-1 host resisted anti-VSV serum. This proportion of pseudotypes was considerably higher than that obtained after infection by *wt* VSV, 0.06%. Clearly the group IV RAV-1 pseudotypes did contain a considerable amount of serologically active VSV G protein but contained a lower relative amount of G protein than did particles formed after *wt* infection.

DISCUSSION

The procedure used here was designed to select for *ts* VSV mutants that, at 39.5°C, are defective specifically in production of functional glycoprotein. It demands that there be little VSV G protein on the cell surface so that the infected cell survives treatment with anti-VSV serum and complement. We do not know, however, exactly how much VSV G present on the surface is needed for complement-mediated cell lysis. Second, it requires that the infected cell accumulate some viral RNA and all viral proteins except G and that the mutant be rescuable by the RAV-1 glycoprotein. The cell must be preinfected by a retrovirus such as RAV-1 so that the retrovirus glycoprotein is incorporated into the budding VSV, forming an infectious VSV(RAV-1) pseudotype (30; Weiss and Bennett, submitted). Of the temperature-sensitive representatives of each of the five VSV complementation groups studied, only *tsO45* (V) was rescuable at the nonpermissive temperature by RAV-1 (Table 1). It was expected that the only *ts* mutants that could survive our selection procedure (growth at 39.5°C in a cell preinfected with RAV-1 in the presence of anti-VSV serum and complement) would be in group V.

How well did this selection procedure work? Of the first 17 workable *ts* mutants that survived the procedure, 4 were in group V. Another five

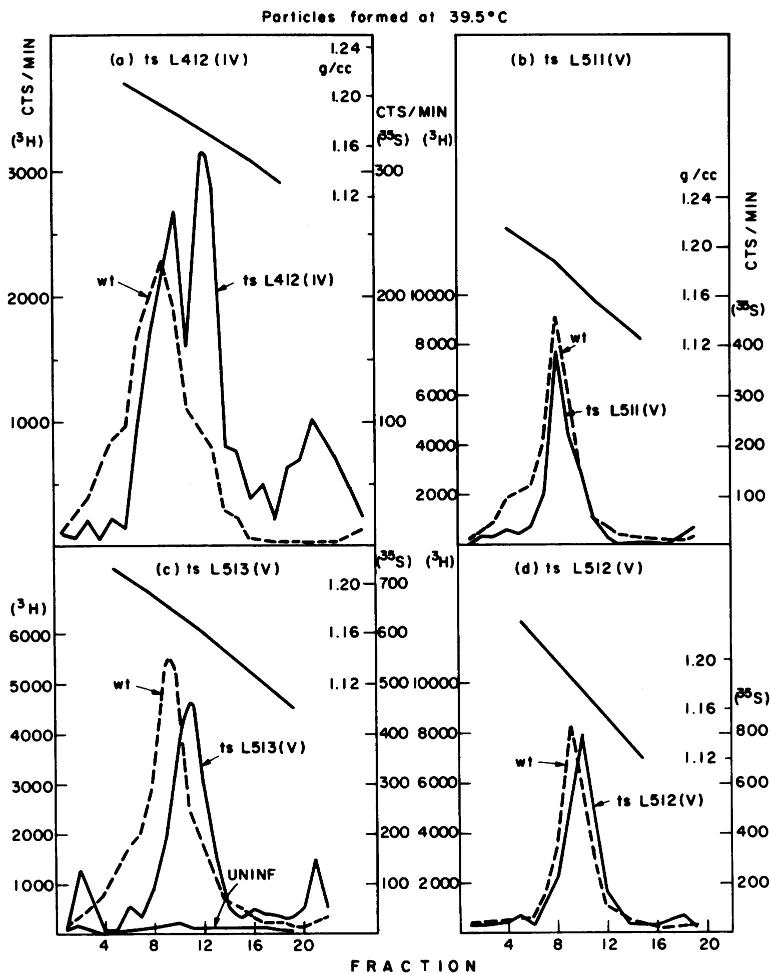

FIG. 3. *Density of defective VSV particules produced at 39.5°C. Vero cells were used, but otherwise the conditions for infection and labeling with [^{35}S]methionine were as in Fig. 2 and Table 2. Mixed with the particulate fraction obtained from the medium was [^{3}H]uridine-labeled wild-type VSV prepared from parallel infections at 39.5°C. Analysis of the material by equilibrium sucrose density gradient centrifugation is detailed in Materials and Methods. Panel (a) is from a separate experiment; note that the density gradient is shallower than in the other three panels. (a) tsL412 (IV); (b) tsL511 (V); (c) tsL513 (V); (d) tsL512 (V). The peak fractions of ^{35}S radioactivity from these fractions were analyzed by SDS-gel electrophoresis. In (b), (c), and (d), there was less than 5% the relative proportion of G protein as in control, wild-type particles; in (a) there was 48%, the control proportion of ^{35}S-labeled G protein.*

were a hitherto undescribed type of group IV mutant that, as we discuss below, results in some defect in production of functional VSV G protein. The other eight mutants induced synthesis of very little VSV protein at 39.5°C and were not studied further. Presumably, they were mutant in one of the genes involved in RNA replication and/or transcription, in addition to any possible defect in G-protein synthesis. In particular, 11 of the 17 workable new *ts* mutants were isolated after FU mutagenesis, and 3 of these were in group V. Considering the extreme rarity of group V mutants in an unselected population of FU-induced *ts* mutants (0 out of 200) (17), we conclude that our procedure indeed enriches for group V mutants but that it is not perfect.

It should be possible to include a second cycle in the selection procedure, which would increase the proportion of G mutants. The population of VSV(RAV-1) pseudotypes that emerge from the initial selection could be grown (on RAV-1-sensitive cells) at 32°C for one cycle, and the resultant virus, now with the VSV glycoprotein coat, could be used to infect RAV-1-producing cells at 39.5°C, with anti-VSV serum and complement added as in the first cycle. Alterna-

TABLE 9. *Rescue of VSV ts mutants at 39.5°C by RAV-1*[a]

Virus	Yield in QT35 cells (PFU/ml)	Ratio of PFU/ml of QT35-RAV-1/QT35	Fraction of QT35-RAV-1 lysate resistant to anti-VSV serum
Wild type	3.0×10^8	0.93	0.0006
*tsG*33 (III)	3.3×10^3	1.10	<0.001
*tsG*41 (IV)	1.4×10^3	0.60	<0.001
*tsO*45 (V)	1.3×10^4	80.0	1.26
*tsL*411 (IV)	3.0×10^5	3.5	0.025
*tsL*412 (IV)	5.5×10^5	4.0	0.036
*tsL*413 (IV)	1.6×10^6	5.1	0.008
*tsL*414 (IV)	4.0×10^5	4.5	0.025
*tsL*511 (V)	1.2×10^5	25	1.07
*tsL*512 (V)	2.7×10^5	10.6	0.30
*tsL*513 (V)	2.1×10^4	25	0.85

[a] Cultures of 2.0×10^6 QT35 or QT35 RAV-1-preinfected (QT35-RAV-1) cells in 35-mm dishes were infected with 4×10^6 PFU of the indicated strains. Incubation was at 39.5°C for 8.5 h. Infectious virus was determined by plaquing on sensitive C/O cells, both before and after treatment with anti-VSV serum. Shown here are: (column 2) the yield from the QT35 infected cell; (column 3) the ratio of yields from QT35-RAV-1 cultures to the QT35 cultures; and (column 4) the fraction of the yield from the QT35-RAV-1 cells that resisted neutralization by anti-VSV serum (see text). Although not detailed here, all of the virus particles released from the QT35 cells were found to be sensitive to neutralization by the anti-VSV serum.

tively, although the VSV(RAV-1) pseudotypes that emerge from the first cycle of selection are not able to reinfect RAV-1-producing cells, they could be used to infect directly at 39.5°C a cell line that is sensitive to RAV-1 but is producing an avian leukosis virus of a different glycoprotein subgroup. Providing this second virus could also rescue *tsG* VSV mutants (as shown for *tsO*45; 27), the antiserum selection could be used on this line, as well, in a second cycle.

New *ts* (V) mutants. Our four new *ts* mutants in group V generally resemble the prototype *tsO*45 (V) (17). Infected cells at 39.5°C induce synthesis of normal amounts of both VSV RNA and protein. Noninfectious particles are formed that contain VSV N and M proteins and presumably VSV RNA, but little, if any, G protein. Noninfectious particles produced by three of the four new mutants, like those produced by *tsO*45 (V) (24), have a density less than that of normal VSV, consistent with the loss of a viral glycoprotein. However, particles formed after infection by *tsL*511 (V) at either 39.5°C (Fig. 3b) or 32°C (not shown) have a normal density. Possibly this means that the *tsL*511 (V) particles contain proportionately more of some cellular membrane protein. Although it is known that some cell surface antigens are incorporated into VSV particles (6), it is not yet known to what extent this happens with any of these noninfectious particles. Virus particles produced at 32°C after infection by *tsO*45 (V), *tsL*511 (V), *tsL*513 (V), or *tsL*514 (V) appear normal with respect to their density, polypeptide composition, and specific infectivity. By contrast, infection at 32°C by *tsL*512 (V) produces a number of light-density, noninfectious, glycoprotein-defective particles (data not shown). Stocks (propagated at 32°C) of most known *ts* mutants in group V are inactivated by heating (9), and the four new *ts* (V) mutants are no exception (Table 8). Thus, the primary *ts* defect in these new mutants appear to be in the structure and/or maturation of the VSV glycoprotein, but further work is necessary to ascertain at what step biosynthesis is affected.

New *ts* mutants in complementation group IV. At first, these mutants appeared paradoxical. In some of their properties they resembled *tsG*41 (IV), a prototype IV mutant, particularly in the absence of accumulation of detectable virus-specific RNA after infection at 39.5°C (Table 6) and in resistance to inactivation by heating (Table 8). In others, they more resemble *tsO*45 (V) and the newly isolated *ts* (V): (i) induction at 39.5°C of high levels of synthesis of VSV proteins (Table 7); (b) formation at 39.5°C of noninfectious or partially infectious particles deficient in VSV glycoprotein (Table 2, Fig. 2 and 3) (no particles were formed after infection at 39.5°C by *tsG*41 (IV) [Table 2]); and (c) enhancement of the yield of virus at 39.5°C by preinfection of host cells by RAV-1 (Table 9) and formation at 39.5°C in an RAV-1-infected cell of a higher than normal percentage of VSV-(RAV-1) pseudotypes (Table 9).

The following explanations are tentative but do serve to interrelate these disparate phenomena. The primary defect in previously studied group IV mutants appears to be in either the N or NS polypeptides which comprise the viral nucleocapsid. Most work supports the contention that the defect is in the structural gene for the N polypeptide (11, 17). This point remains controversial (2), but it does not affect the propositions considered below. We imagine that the same is true of our new *ts* (IV) mutants, and that this defect in nucleocapsid accumulation is expressed, initially, in reduced synthesis of viral genome RNA and all viral mRNA's (5, 25). As a consequence, all viral proteins are formed, but in reduced amounts. One possibility is that the reduced amount of G protein formed in these cells at 39.5°C will mature normally to the cell

surface. However, the density of glycoprotein in the plasma membrane might be too low to allow normal budding of infectious particles from the (reduced) level of nucleocapsid in the cell; as a consequence, the VSV particles that emerge from the cell are relatively deficient in G protein and are noninfectious. Alternatively, a sufficient amount of G protein might mature to the cell surface in cells infected at nonpermissive temperatures by these *ts* group IV mutants. However, the proper lateral clustering or conformation of the surface G protein might be dependent on the presence of a functional viral nucleocapsid, and this interaction could be defective in cells infected by these mutants. Supporting this notion is previous work showing that binding of matrix protein to the plasma membrane did not occur at 39.5°C in cells infected by *ts*G41 (IV); apparently, binding of M protein requires both VSV G protein and nucleocapsid (10). In either case, if the host cell is preinfected by the leukosis virus RAV-1, considerable RAV-1 glycoprotein will be present on the cell surface. Thus, RAV-1 glycoprotein would supplant the VSV G protein on the surface and would be incorporated into budding VSV particles, thereby increasing their infectivity (Table 9). These particles appear to be mosaic, containing both VSV G and RAV-1 glycoproteins, since their infectivity can be reduced substantially by anti-VSV serum (Table 9). Thus, infection of RAV-1-producing cells by the new *ts* (IV) mutants results in a higher proportion of anti-VSV-resistant RAV-1 pseudotypes among the progeny than after infection by *wt* VSV, but lower than after infection by *ts* (V) mutants, where there is no VSV G protein on the surface (10, 11, 13).

Extension to other virus systems. Formation of viral pseudotypes (the genome of one virus enclosed by the glycoprotein of another) is extremely common among lipid-containing viruses (29). Providing one has antiserum that is totally specific for one of the two partners in a pseudotype, the same technique used here should allow the isolation of *ts* mutants in the glycoprotein gene of many lipid-containing viruses.

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