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Envelope Proteins and Replication of Vesicular Stomatitis Virus: In Vivo Effects of RNA+ Temperature-Sensitive Mutations on Viral RNA Synthesis

CLAIRE MARTINET,* ARLETTE COMBARD, CHRISTIANE PRINTZ-ANÉ, AND PIERRE PRINTZ

Institut de Microbiologie, Université de Paris-Sud, Centre d'Orsay, 91405 Orsay Cedex, France

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Temperature-sensitive (*ts*) mutants of vesicular stomatitis virus belonging to complementation groups III and V were investigated for their in vivo RNA synthesis. The sucrose gradient patterns of the RNA species which they produced at nonpermissive temperature (39.2°C) were systematically compared under different experimental conditions: variation of input multiplicity and of time of infection, superinfection with T particles, and temperature shifts. Finally, a more precise analysis of the various RNA species synthesized was carried out. It appeared that the characteristics of RNA synthesis specified at 39.2°C by *ts*III or *ts*V mutants differed from the normal RNA synthesis of vesicular stomatitis virus wild type. Their common depression at 39.2°C in virion-like RNA (38S) production—i.e., so-called genome replication—was tentatively paralleled with the concomitant *ts* events which have been previously shown to affect the two viral envelope proteins. An overproduction of the RNA transcripts was described for mutants in group III and posed the question of a regulation process to determine the amount of RNA to be transcribed.

In the process of elucidating the virus-specified events in cells infected with vesicular stomatitis virus (VSV), much interest has been centered on temperature-sensitive (*ts*) mutants of this virus. Up to now, information about mutants classified as RNA+ (able to make a readily detectable amount of actinomycin D-resistant RNA) was almost limited to description of the protein synthesis they induced at restrictive temperature (21, 32). Indeed, one may a priori assume that their RNA syntheses would be fully comparable to those of the wild type and therefore that these groups are rather altered in a maturation step late in the viral cycle. In fact, defects in envelope proteins M and G have been demonstrated for groups III and V, respectively (4, 9, 11, 12, 33).

Despite the sparseness of published results about the RNA synthesis induced by *ts*III and *ts*V mutants, at least one observation prompted us to characterize in more detail their intracellular RNA species: the abnormally high level of RNA synthesis obtained in *ts*III-infected cells at restrictive temperature, either in cumulative measurement (20) or in a sucrose gradient pattern (29). Therefore, a comparative analysis of the RNA syntheses induced by *ts*III and *ts*V mutants was systematically conducted, and an alteration in their syntheses was demonstrated.

MATERIALS AND METHODS

Cells and viruses. All the analyses of RNA syntheses in vivo were performed on HeLa cell monolayers grown as already described (22) and on chicken embryo cultures when a low level of residual cellular protein synthesis was required.

VSV (Indiana serotype) strains investigated were: a wild type which gives twice as many plaques at 30 as at 39.2°C, parent of the mutants; *ts* mutants belonging to complementation group III [*ts*O23(III), *ts*O76(III), and *ts*O89(III)], and *ts* mutants belonging to complementation group V [*ts*O45(V), *ts*O44(V), and *ts*O110(V)]. All mutant stocks, containing only standard B particles, were checked for low percentage of revertants and low residual growth at 39.2°C and by complementation tests as reported previously (22).

Defective interfering T particles were prepared by infecting chicken embryo monolayers with a third high-multiplicity passage of wild-type VSV. After a low-speed centrifugation to remove cells and debris, the virions (B and T particles) were pelleted at 33,000 rpm for 1 h at 4°C in a Spinco 45 rotor. The pellets were suspended in a saline buffer (0.01 M Tris [pH 7.4], 1 M NaCl, 1 mM EDTA). The suspension was layered onto a 5 to 40% sucrose gradient in the same buffer and centrifuged (18,000 rpm for 1.5 h at 4°C in a Spinco SW25 rotor). The band of T particles was removed by suction, and the yield of T particles in the preparation was calculated in equivalent PFU from optical density at 260 nm (7). The T particles were checked for homogeneity in size, corresponding to short T particles with 19S RNA.

Sucrose gradient fractionation of uridine-labeled products. HeLa cells were infected at the multiplicity specified for each experiment and treated with actinomycin D. After labeling with [^{3}H]uridine (20 μCi/ml), cytoplasmic extracts were prepared as already described (22). From this starting material two different analyses could be conducted. (i) RNA species were solubilized from the cytoplasmic extract by addition of sodium dodecyl sulfate to a final concentration of 1%; they were fractionated by centrifugation in a 15 to 30% sucrose gradient at 21,000 rpm for 15 h at 17°C in a Spinco SW27.1 rotor, as described elsewhere (2). (ii) The cytoplasmic extract was also fractionated on a 15 to 30% sucrose gradient in high saline buffer (0.01 M Tris [pH 7.4], 0.5 M NaCl, 0.05 M $MgCl_2$ at 27,000 rpm for 3 h at 4°C in the Spinco SW27.1 rotor. Determination of the radioactivity in an aliquot of each fraction collected enabled us to locate the polysomes (positioned on both sides of the 120S nucleocapsid peak) and the free mRNA present as ribonucleoprotein structures migrating just above the ribosomal optical density peak. Fractions were pooled (polysomes on the one hand and free mRNA on the other); the RNA was phenol extracted (22), and polyadenylic acid-containing mRNA's were isolated according to their affinity for oligodeoxythymidylic acid [oligo(dT)]-cellulose as described below. The mRNA's were analyzed on a 15 to 30% sucrose gradient in sodium dodecyl sulfate as usual.

Analyses of viral RNA. After separation on sucrose gradients, the various VSV RNA species were analyzed in several ways.

(i) Hybridization. Samples of [^{3}H]RNA melted at 100°C for 2 min were hybridized with unlabeled RNA extracted with phenol from purified virions. Annealing was conducted in 0.01 M Tris-0.5 M NaCl, pH 7.4 (final volume, 40 μl), in the presence of 0.5 μl of diethylpyrocarbonate. Annealing was performed at 70°C for at least 24 h. Then the amount of RNase-resistant radioactivity in each annealed sample was determined after a 30 min digestion with pancreatic RNase A (10 μg/ml, final concentration).

(ii) Melting. Samples of [^{3}H]RNA were heated in 90% dimethyl sulfoxide for 4 min at 60°C to separate any possible complexed RNA strands from each other (31). Then RNAs were recovered by precipitation with 2 volumes of ethanol in the presence of 0.2 M NaCl and separation on a 15 to 30% sucrose gradient as described above.

(iii) Binding to oligo(dT)-cellulose. Samples of [^{3}H]RNA were mixed with oligo(dT)-cellulose (0.2 g) in 0.01 M Tris-hydrochloride (pH 7.4)-0.5 M KCl. The reaction began simply as a batch kept for 15 min at room temperature; then the suspension was poured into a small column, and the first effluent was passed twice more over the column. The column was then washed with about 4 ml of 0.01 M Tris-0.5 M KCl while a slow flow rate was maintained throughout. Adsorbed RNA was eluted with about 1 ml of 0.01 M Tris. The measurement of the acid-precipitable radioactivity of each fraction made possible the selection of appropriate fractions, which were pooled and eventually analyzed on a 15 to 30% sucrose gradient.

(iv) Methylation. Infected cells were maintained at the appropriate temperature (30 or 39.2°C) for 2 h in Eagle minimal essential medium with only 10 μM methionine but supplemented with 10 μg of actinomycin D per ml and 2% fetal calf serum. At 2 h postinfection (p.i.), cells were labeled with 10 μM [*methyl*-^{3}H]methionine (160 μci/ml) in the above medium without cold methionine but in the presence of 10 mM sodium formate and 10 μM guanine and adenine. In parallel, cultures were labeled with [^{3}H]uridine (20 μCi/ml) in the same medium adjusted to 10 μM with methionine. At 4 h p.i., cells were scraped off the sides of the flasks and treated with proteinase K (100 μg/ml) for 1 h at 37°C, and RNA was twice extracted with phenol-chloroform and then alcohol precipitated.

Analysis of viral proteins. Conditions for infection, labeling of viral proteins in chicken embryo cells, and treatment of the cytoplasmic extract before electrophoresis, as well as the electrophoretic procedure, were exactly the same as previously described (2).

Chemicals. Additional compounds, not mentioned previously (2, 3, 22), were dimethyl sulfoxide, from E. Merck AG, Darmstadt, Germany; oligo(dT)-cellulose, from Collaborative Research Inc., Waltham, Mass.; and diethyl pyrocarbonate, from Hopkins & Williams, Chadwell-Heath, England. Actinomycin D was a generous gift from Merck Sharp & Dohme, West Point, Pa. Radioisotopes were obtained from the Commissariat à l'Energie Atomique, Saclay, France.

RESULTS

Results reported below refer to mutants *ts*O23(III) and *ts*O45(V). It must be pointed out that within each of these complementation groups only one phenotype has been constantly observed, whatever the mutant considered: *ts*O23(III), *ts*O76(III), or *ts*O89(III) and *ts*O45(V), *ts*O44(V), or *ts*O110(V).

Characteristics of RNA synthesis induced at 39.2°C by mutants of groups III and V. (i) Global pattern in sucrose gradient. A uniform labeling from 1 to 4 h p.i. with [^{3}H]uridine was applied to equivalent batches of cells infected in duplicate with *ts*O23(III) or *ts*O45(V) and maintained at either 30 or 39.2°C. Cytoplasmic viral RNAs were analyzed by sucrose velocity gradients. At 39.2°C both *ts*O23(III) and *ts*O45(V) synthesized all the characteristic RNA species normally seen at the permissive temperature, but with different proportions (Fig. 1). The levels of synthesis at 39.2°C of the 13-15 and 28S RNAs were approximately the same as those at 30°C. Some increase at high temperature could even be detected in the 13-15S peak. Conversely, the synthesis of the 38S RNA seemed to be depressed at 39.2°C. These modifications are characteristic of *ts*O(III) and *ts*O(V) mutants; in the case of infection with wild-type VSV, the RNA profiles were only slightly modified with the increase of temperature (data not shown).

(ii) Effect of input multiplicity on RNA

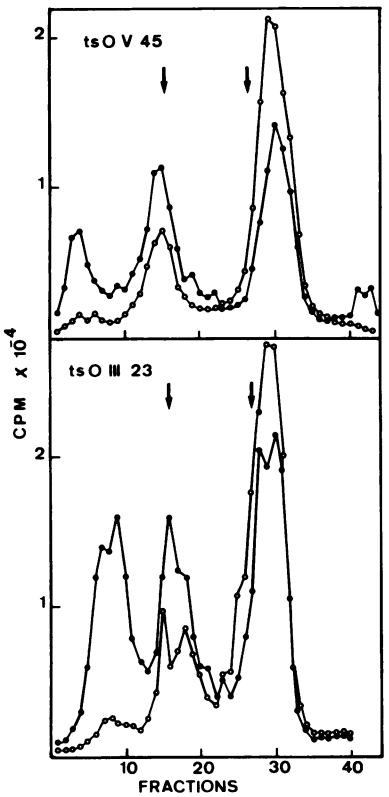

FIG. 1. *In vivo RNA synthesis directed by tsO23(III) and tsO45(V). HeLa cells were infected for 45 min at room temperature with either tsO23(III) or tsO45(V) (100 PFU per cell). Then they received complete Eagle essential medium with 10 μg of actinomycin D per ml, [³H]uridine (20 μCi/ml) from 1 to 4 h p.i. Cells were washed, scraped off the flasks, pelleted, and disrupted in reticulocyte standard buffer. Then sodium dodecyl sulfate was added (1%[wt/vol] final concentration) to solubilize cytoplasmic RNAs, which were then analyzed by velocity centrifugation in a 15 to 30% sucrose gradient with sodium dodecyl sulfate at 21,000 rpm for 15 h in the Spinco SW27.1 rotor at 17°C. Arrows on the figure show the peak positions of the 18 and 28S ribosomal markers from HeLa cells. Symbols: ●, 30°C; ○, 39.2°C.*

synthesis. The influence of the multiplicity of infection on an extended labeling as above (1 to 4 h p.i.) was investigated for both groups of mutants. By summing the total radioactivity within each of the three main peaks in the gradient profiles, we could evaluate the ratio of the RNA induced at 39.2°C to that induced at 30°C and follow its variation versus the multiplicity of infection (Fig. 2). The ratio for our wild-type strain, being independent of the multiplicity over a wide range, was taken as a reference (equal to ≃0.6). Strikingly, for both group III and group V mutants the ratios were strongly multiplicity dependent, and they became distinctly higher than the wild-type ratio above the moderate multiplicity values that we usually used (50 PFU per cell). Looking to the corresponding profiles, we observed that the overproduction here illustrated at 39.2°C was fully due to excessive synthesis of the so-called mRNA's (see, e.g., Fig. 1), especially for the *ts*III mutant. In all cases the 38S peak was much more moderate at 39.2 than at 30°C and never accounted for the observed increase of the 39.2/30°C ratio. However, the amount of 38S RNA rose as the multiplicity was increased.

(iii) Effect of time on RNA synthesis. Since a long labeling period represents a steady-state condition, shorter pulses were then performed. At different times of the infection cycle, [³H]-uridine was added to the medium, and the total resulting labeling in each RNA category was measured as the area of each gradient peak. This allowed a ready comparison of the hourly rates of synthesis of 38, 28, and 13–15S at 30 and 39.2°C by each of the two mutants (Fig. 3). The curves obtained at 30°C for 13–15S illustrated the well-known predominance of the mRNA's during the first stages of the normal cycle (24, 26). The 38S RNA emerged more slowly and culminated rather late, just before being released as part of the virions. Because of their similarity, results obtained at 39.2°C with either *ts*O23(III)

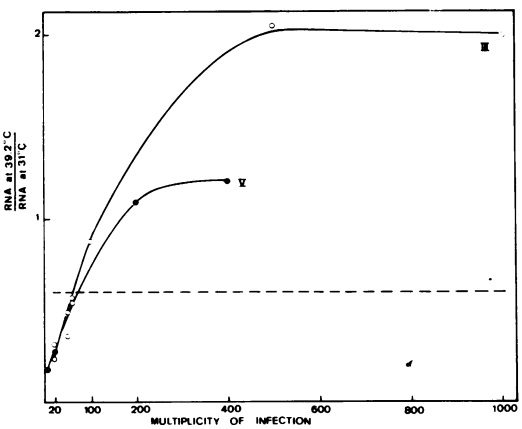

FIG. 2. *Effect of multiplicity of infection on total RNA synthesis by mutants from groups III and V. For each multiplicity of infection used, the amount of viral RNA was calculated at either 30 or 39.2°C by summing the total radioactivity of the three main peaks observed in a sucrose gradient profile as in Fig. 1. The evaluated ratio of counts per minute at 39.2°C to counts per minute at 30°C was then plotted versus the multiplicity of infection. Symbols: ○, tsO23(III); ●, tsO45(V). The broken line corresponds to the value observed for a wild-type strain close to the strain from which the mutants were isolated and which was not completely heat-resistant.*

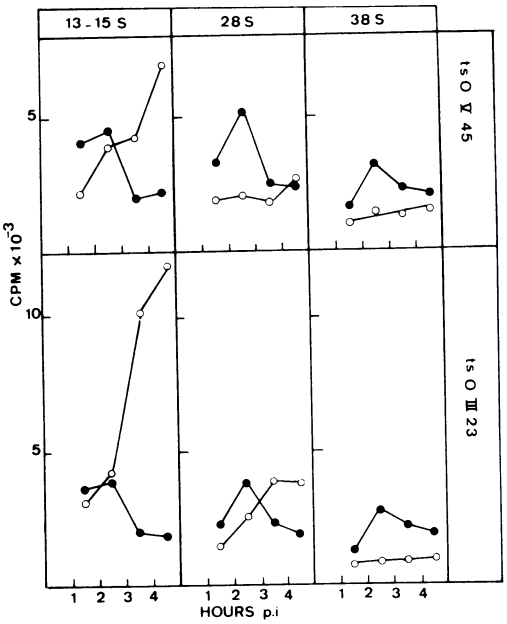

FIG. 3. *Effect of time on the synthesis of each RNA species. HeLa cells were infected with either tsO23(III) or tsO45(V) (multiplicity of infection ≃ 100 PFU per cell). After adsorption at room temperature, the cells received complete Eagle medium supplemented with actinomycin D. For each mutant, two series of monolayers were used: one was incubated at 30°C, and the other was incubated at 39.2°C. [^{3}H]uridine (20 μCi/ml) was added at different times of infection, and cytoplasmic extract was prepared after 1 h of labeling. RNAs were solubilized and layered onto linear 15 to 30% sucrose gradients. The total radioactivity was calculated for each peak observed after gradient fractionation and was plotted at the time value corresponding to the mean time of the uridine pulse considered. Symbols: ●, 30°C, ○, 39.2°C.*

or *ts*O45(V) may be now discussed together. During the first hours (up to 2 to 3 h p.i.), the synthesis of all viral RNAs was impaired at 39.2°C without a selective inhibition of any peak and thus led to a qualitatively normal RNA profile. Later in the cycle, this normal equilibrium between the three main RNA peaks became progressively altered in the following way. First, no increased rate could be observed in 38S formation: this RNA appeared merely at a constant restricted rate. On the contrary, mRNA's were synthesized with increasing speed and became several times more important than they were at permissive temperature. One may notice the different slopes of the curves of the 13–15S RNA, which showed again that mutant *ts*O23(III) synthesized larger amounts of this RNA than did mutant *ts*O45(V).

Question of a replicative defect in *ts*III and *ts*V mutants. The observations reported above have demonstrated the deficient amount of 38S RNA produced at 39.2°C by either *ts*III or *ts*V mutants. The possibility of an actual defective step in the replication process of the viral genome will now be examined.

(i) Superinfection with T particles. Interference with the production of viral standard B particles of VSV by its T particles is thought to occur via a competition at the level of the replication of the two types of genomes (7, 16, 18). This is characterized in viral RNA synthesis by the appearance in the gradient profile of a predominant 19S peak, yielding new T-particle genomes (26). Such a deviation is shown in the control (wild type or mutants at permissive temperature) panel of Fig. 4. In this experiment the superinfection with T particles was done as indicated by Stampfer et al. (26): T particles were added 2.5 h after the B particles at an equivalent multiplicity to that of the B particles already adsorbed. Cells were exposed to [^{3}H]uridine from 3 to 6 h p.i. We did not observe (Fig. 4) any change at all in the 39.2°C RNA profiles of *ts*O23(III) or *ts*O45(V), and no inhibition of the exaggerated 13–15S RNA synthesis or accumulation of T genomes could be detected, even when the resolution of the gradient was improved by extending the time of centrifugation (bottom panels). Therefore, at nonpermissive temperature the replication of T particles was prevented in *ts*O23(III)- or *ts*O45(V)-infected cells. This result argues for an alteration of the replicative process of these mutants. The two following experiments were performed to find out which possible step could be impaired.

(ii) Melting of 28S RNA material. A first explanation of the restricted appearance of the genome-like RNA among the RNA synthesized by *ts*O(III) or *ts*V mutants was a piling up of most of the 38S RNA. This might occur by prevention of its leaving the pool of replicating and transcribing complexes present in the 28S region of a gradient (25, 26). Therefore, after a labeling from 2 to 5 h p.i., cytoplasmic extracts from cells infected with either *ts*O23(III) or *ts*O45(V) were fractionated in sucrose gradients as usual. The fractions sedimenting in the 28S region were pooled and separated into two aliquots, one of which was melted in 90% dimethyl sulfoxide before being ethanol precipitated, as was done directly for the other. Both precipitated RNAs were analyzed in 15 to 30% sucrose gradients (Fig. 5). As a control, the 28S RNAs synthesized at 30°C by each mutant showed after melting the same proportion of radioactive 38S RNA as is normally present in such material. In case of infection at 39.2°C by either mutant, the melting procedure released essen-

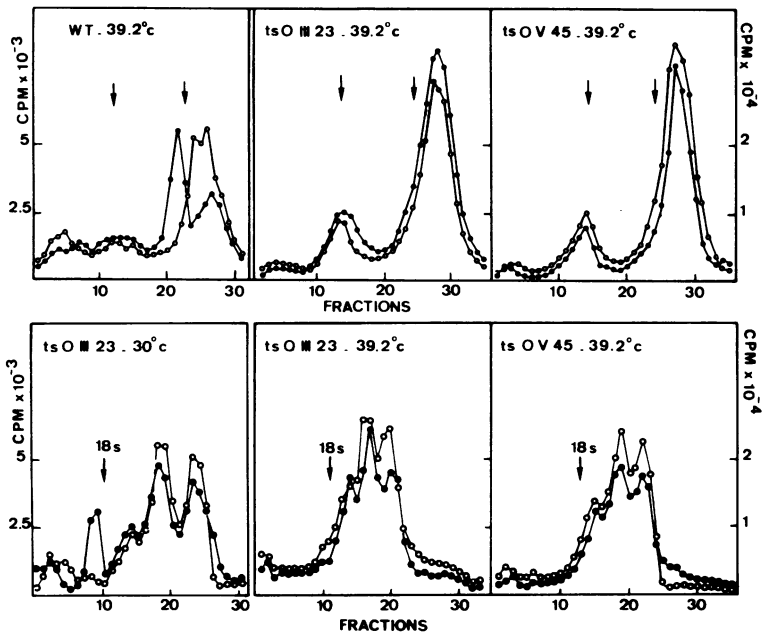

FIG. 4. *Sucrose gradient patterns of the RNA synthesized in ts mutant-infected cells superinfected with VSV T particles. HeLa cells were infected with either tsO23(III), tsO45(V), or wild-type (WT) B particles (multiplicity of infection ≃ 50 PFU per cell) and incubated at 39.2°C. After 2.5 h p.i. the medium was discarded and the cells were superinfected with defective interfering T particles at an equivalent multiplicity to that of the B particles; half of the cultures were parallely mock infected with saline buffer. Thirty minutes later, the inoculum was substituted with medium containing actinomycin D. At 3 h after the first infection, [³H]uridine was added, and cells were harvested at 6 h p.i. Cytoplasmic extracts were processed as for Fig. 1. In the case of the three bottom panels, the RNAs were analyzed in the same sucrose gradient but at 33,000 rpm for 17 h in a Spinco SW41 rotor at 17°C. Symbols: ○, single infection; ●, superinfection with T particles.*

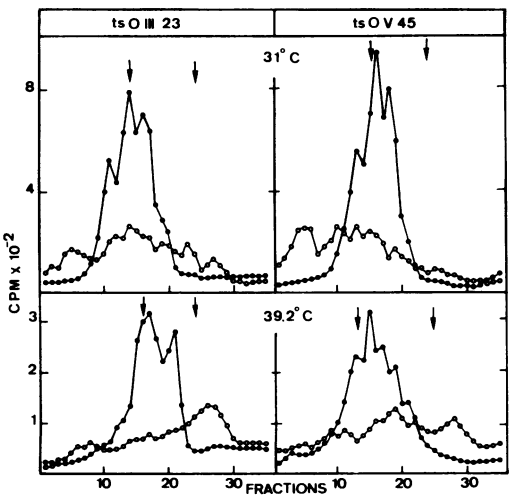

FIG. 5. *Analysis of products obtained by melting 28S RNAs. Viral RNAs induced by tsO23(III) or tsO45(V) and labeled with [³H]uridine from 2 to 5 h were analyzed as indicated in Fig. 1. After localization of the 28S peak by precipitating aliquots along the gradient with trichloroacetic acid, the fractions*

tially light RNAs (i.e., <38S) and not—as could be expected—an increased amount of 38S RNA. The significance of this result could be reinforced when the RNA complexes recovered in the 28S region were shown not to be artifacts arising from a random association during our whole experimental procedure. Indeed, addition of radioactive 38S RNA extracted from VSV purified virions to cold VSV-infected cells just at the time when they were scraped into reticulocyte standard buffer before being submitted to the usual extraction-fractionation procedure did not lead to the appearance of radioactivity in

of this area were pooled and separated in duplicate: one was directly precipitated by addition of 2 volumes of ethanol, whereas the other first received dimethylsulfoxide to a final concentration of 90% and was melted at 60°C for 4 min. Both aliquots were suspended in reticulocyte standard buffer–1% sodium dodecyl sulfate and analyzed on sucrose gradients as usual. Cold ribosomal markers were added to each gradient. Symbols: ●, original 28S RNA; ○, melted 28S RNA.

the 28S region of a 15 to 30% sucrose gradient. From this experiment, the hypothesis of some hidden 38S RNA within the 28S complexes has to be ruled out.

(iii) Polarity of RNAs synthesized at 39.2°C by *ts*O23(III) and *ts*O45(V). If the replicative defect demonstrated above for both group III and group V mutants consisted in a deficient 38S genome completion, accumulation of negative strands could be expected in other regions of the gradient. Therefore, the polarity of the molecules present in each radioactive peak detected at 39.2°C was determined by annealing them with unlabeled virion genome, after melting of the radioactive material. The comparison (Table 1) of the negative-strand yields thus obtained with the normal ones (i.e., in the wild type at 30 or 39.2°C or in each mutant at 30°C) did not reveal any special accumulation at 39.2°C of negative strands in each RNA category of the 15 to 30% sucrose gradient, whatever the mutant used. In fact, more 38S RNA could be annealed to viral genome than normally when it had been synthesized at 39.2°C. But this was not really significant, because—despite the special care taken to avoid contamination with short nascent RNA strands—the 38S RNA analyzed presented about 11% self-annealing.

Effects of temperature shifts. The use of temperature shifts demonstrated successfully that the G and M proteins behave abnormally at 39.2°C during the maturation of *ts*O23(III) and *ts*O45(V), respectively (11, 12). Therefore, similar temperature shifts were imposed on *ts*O45(V)- or *ts*O23(III)-infected cells, and the viral RNA syntheses were then analyzed. For both mutants, we compared (Fig. 6) the profiles of the RNAs labeled from 5 to 6.5 h p.i. under three different conditions: (i) labeling at 30°C in cells maintained at this temperature throughout, (ii) labeling at 39.2°C in cells which had been transferred from 30 to 39.2°C at 2.5 h p.i., and (iii) labeling at 30°C in cells which had just been incubated back at 30°C after a first shift from 30 to 39.2°C. Attention has to be paid to the difference of the scale used for each mutant. Compared to normal 30°C profiles (which were very similar for both mutants), the simple shift from 30 to 39.2°C induced a marked change in the RNA profiles of both mutants, where the alterations already described were particularly significant: an evident disequilibrium took place which favored the transcribed RNAs at the expense of 38S RNA accumulation (especially weak). In the case of *ts*O23(III), the 13-15S peak could amount to as much as 20 times its normal yield, even by 1 h after the temperature shift.

TABLE 1. *Annealing of intracellular viral RNAs with virion RNA*[a]

Virus	Type of labeled RNA	RNase resistance (%)[b]		
		Before annealing	After annealing with genome RNA	Self-annealing
Wild type at 30 or 39.2°C or mutants at 30°C	13-15S	7 ± 10	99 ± 8	9 ± 6
	28S	12 ± 8	88 ± 10	26 ± 5
	38S	2 ± 5	26 ± 7	30 ± 4
*ts*O23(III) at 39.2°C	13-15S	2 ± 3	100 ± 5	8 ± 5
	28S	13 ± 5	100 ± 9	21 ± 7
	38S[c]	11 ± 7	40 ± 2	11 ± 9
*ts*O45(V) at 39.2°C	13-15S	9 ± 2	89 ± 8	10 ± 14
	28S	15 ± 3	78 ± 12	22 ± 5
	38S[c]	7 ± 5	35 ± 7	16 ± 6

[a] After extraction and isolation of ^{3}H-labeled viral RNAs as described in Fig. 1, material from each peak (at least 1,500 cpm) was mixed with unlabeled RNA extracted from VSV virions. Annealing was allowed at 70°C for at least 24 h, and the proportion of radioactivity rendered resistant to a 30-min digestion with pancreatic RNase was estimated by acid precipitation. The excess of genome RNA was checked by hybridization of increasing levels of this one with total labeled infected-cell RNA. The value obtained for *ts*O23(III)-infected cells at 39.2°C being the highest, it was chosen as a basis to calculate the quantity of genome RNA to be added in each hybridization sample (≃3 μg).

[b] Mean values of several experiments.

[c] The 38S peak was always contaminated with short nascent RNAs essentially of positive polarity. In the case of the poorly defined 38S RNA peak observed during analysis of the RNA synthesized at 39.2°C by either mutant, this contamination was especially inconvenient for subsequent characterization. Therefore, the fractions corresponding to the 38S zone in a first gradient were pooled, melted in 90% dimethyl sulfoxide, and then fractionated on a second 15 to 30% sucrose gradient. The only molecules migrating again as 38S material were annealed.

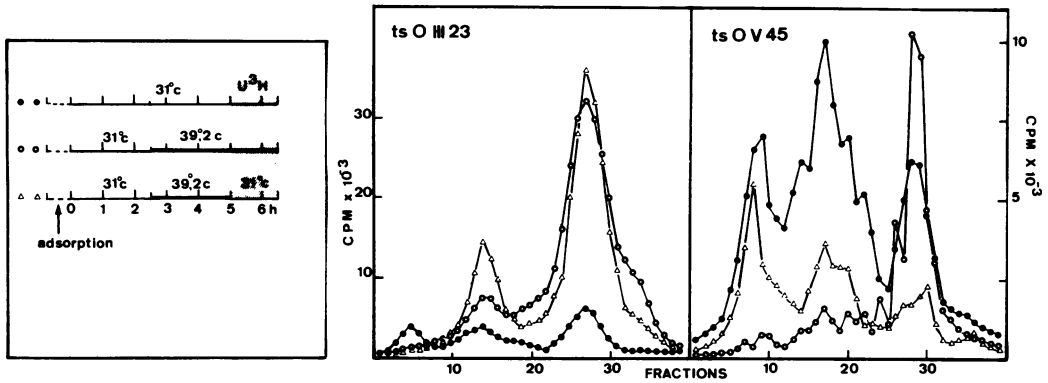

FIG. 6. *RNA species synthesized by tsO23(III)- and tsO45(V)-infected cells under different temperature conditions. After infection with tsO23(III) or tsO45(V), actinomycin D-treated cells were incubated at 30°C. At 2.5 and then at 5 h p.i., the cells were shifted up to 39.2°C and then down to 30°C as indicated in the left panel. All samples were labeled with [³H]uridine (20 μCi/ml) from 5 to 6.5 h p.i. Cytoplasmic extracts were prepared and analyzed as in Fig. 1. Symbols: ●, permissive temperature; ○, upward shift conditions; △, double temperature shift.*

By extending the time centrifugation of the gradient, the various RNA species in the 13–15S peak could be resolved as three distinct peaks (i.e., 17, 15, and 11S), which had the same increase (Fig. 4, bottom panels). Except for this difference in magnitude of RNA production after the shift, the profiles for both mutants were rather similar. On the contrary, the two mutants completely differed in the case of a double temperature shift. In *ts*O23(III)-infected cells, altered RNA synthesis persisted despite the return to permissive temperature. Conversely, when shifted back to 30°C, the *ts*O45(V)-infected cells led to a rapid change towards a new unbalance between the three peaks of the RNA profile, yielding a tremendous amount of 38S RNA at the expense of the 13–15S RNA. An actual burst in 38S RNA led to the normal level of 38S accumulation within 1.5 h.

Overproduction of RNA by *ts*III mutants at 39.2°C. The temperature shiftup just showed more dramatically the excessive synthesis of 28 and 13–15S RNAs by *ts*O23(III) at 39.2°C, as described above on several occasions. The question arises whether this really concerned the transcription process, which would be exaggerated owing to the defect of group III mutants. First, the possibility was ruled out that the increase of radioactivity in the 28 and 13–15S species made at 39.2°C was simply due to an increase of the specific radioactivity, as that could be suspected with regard to the abnormal effect of group III mutants on the permeability of chicken embryo cells to uridine (5) at 39.2°C. In fact, this phenomenon was not observed on HeLa cells (5; personal observation); furthermore, by extending the spin time (as for Fig. 4), the viral RNAs in 13–15S peak could be separated from the 18S ribosomal RNA, and the ratio of counts per minute to optical density was found to be identical for viral RNA peaks at both 30 and 39.2°C (data not shown). Therefore, at 39.2°C the mutant *ts*O23(III) actually induced the synthesis of an excess number of 13–15S, as of 28S, molecules. They were then examined for their putative messenger character.

(i) Analyses of 28 and 13–15S RNAs. One of the characteristics of VSV mRNA's is their complementarity to the virion genome. In Table 1, one can see that the positive polarity of 28 and 13–15S RNAs induced at 39.2°C by *ts*O23(III) was consistent with this criterion. As a second characteristic, the presence of polyadenylated tails was checked by chromatography on oligo(dT)-cellulose, and the amount of polyadenylic acid-containing RNAs in the 28 and 13–15S material recovered at 39.2°C (from 1 to 3 h p.i.) was compared with the normal amount observed at 30°C. Virtually no difference in these two proportions was revealed (Fig. 7). One may conclude from these two results that the overproduction of 28 and 13–15S RNA by *ts*O23(III) at 39.2°C was related with the process of transcription.

(ii) Translation. To see whether an excess of protein synthesis took place concomitantly, cells were infected with *ts*O23(III) under the same conditions as in Fig. 7, and viral protein synthesis was followed by incorporation of [³H]leucine (Fig. 8). Approximately the same amount of each viral polypeptide (except for the L protein, but this is a general feature of all mutants) was translated at 30 and 39.2°C, despite the double amount of available mRNA observed at restric-

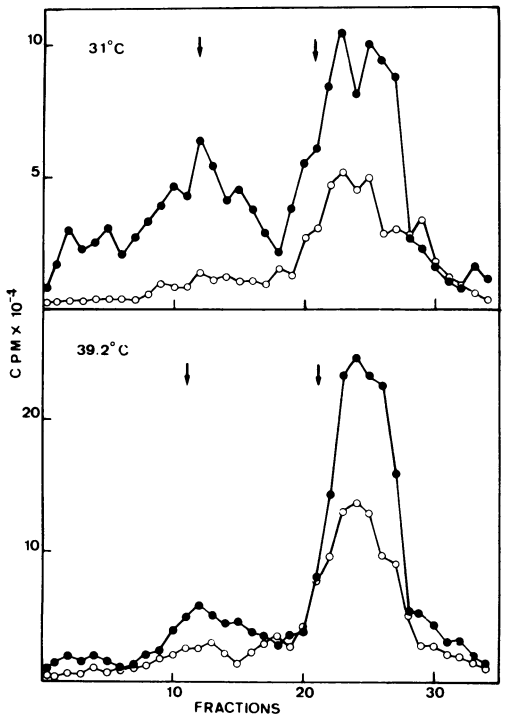

FIG. 7. *Sucrose gradient analysis of polyadenylated RNA synthesized by tsO23(III) at 30 and 39.2°C. Total cytoplasmic RNAs from cells infected with tsO23(III) at 30 or 39.2°C were each divided into two aliquots. One aliquot was directly analyzed by sucrose gradient centrifugation; the other one was loaded onto an oligo(dT)-cellulose column to adsorb the RNA containing polyadenylated tracks. After elution, these RNAs were analyzed in comparison with their counterparts. Symbols: ●, total RNA; ○, polyadenylic acid-containing RNA.*

tive temperature in this experiment. This seems to be correlated with the distribution of the mRNA molecules within the cytoplasm. Indeed, two pools of viral mRNA have been demonstrated: (i) polysomal and (ii) free mRNA in ribonucleoprotein structures (6). Therefore, we prepared both fractions from *ts*O23(III)-infected cells as described in Materials and Methods. From the RNAs extracted from both fractions, mRNA's were separated according to their binding to oligo(dT)-cellulose. Results (Table 2) obtained at 30°C showed the normal distribution of RNA transcripts: about 20% were trapped as polysomes, whereas most of the molecules remained free in the cytoplasm, in accordance with Huang et al. (6). This proportion was not preserved in the case of infection with *ts*O23(III) at 39.2°C: no more mRNA was found as polysomal RNA, but the amount of free mRNA was twice that observed at 30°C. As methylation of viral mRNA seems to be an important factor for their subsequent translation, at least in vitro (1, 28), we checked the extent of methylation of the mRNA synthesized at 39.2°C by *ts*O23(III) (Fig. 9). Whereas in this experiment the mRNA's

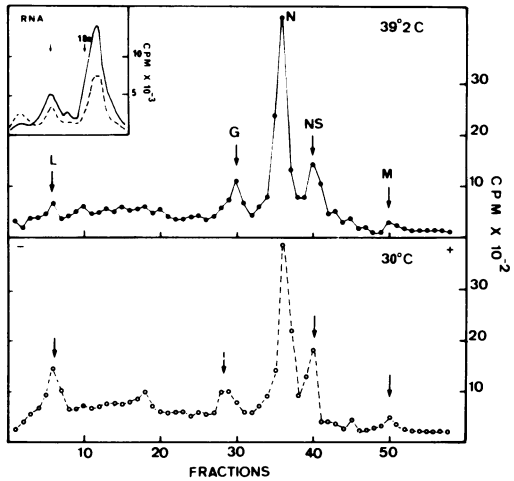

FIG. 8. *Synthesis of virus-specified proteins in cells infected with tsO23(III). Monolayers infected with tsO23(III) were covered with 1/10 (vol/vol) diluted minimal essential medium and incubated at either 30 or 39.2°C. [³H]leucine (10 μCi/ml) was added 1.5 h later. Cells were harvested at 3 h p.i. Proteins were extracted from the cytoplasmic content of disrupted cells by boiling with 10% acetic acid-0.5 M urea-1% sodium dodecyl sulfate-0.1% 2-mercaptoethanol. Extracted ³H-proteins were subjected to electrophoresis in 7.5% neutral sodium dodecyl sulfate-acrylamide gels along with ¹⁴C-proteins extracted from purified virions as markers (arrows). The insert shows the RNA syntheses under the same conditions at 30°C (- - -) or 39.2°C (——).*

TABLE 2. *Location of mRNA in tsO23(III)-infected cells*[a]

Location	Temp (°C)	cpm	
		Total	Oligo(dT)-cellulose bound
Polysomes	30	19,600	1,400
	39.2	12,000	1,300
Free ribonucleoprotein	30	74,000	6,900
	39.2	153,000	13,500

[a] Cytoplasmic extracts from *ts*O23(III)-infected cells at 30 and 39.2°C were fractionated on 15 to 30% sucrose gradients in high saline buffer (27,000 rpm, 3 h, 4°C in the Spinco SW27 rotor). Fractions lighter than the 80S ribosomal peak and fractions of the polysomal area were pooled separately, and their RNA was extracted according to the phenol method. Their content in mRNA was estimated by the amount of RNA bound to oligo(dT)-cellulose as in Fig. 8.

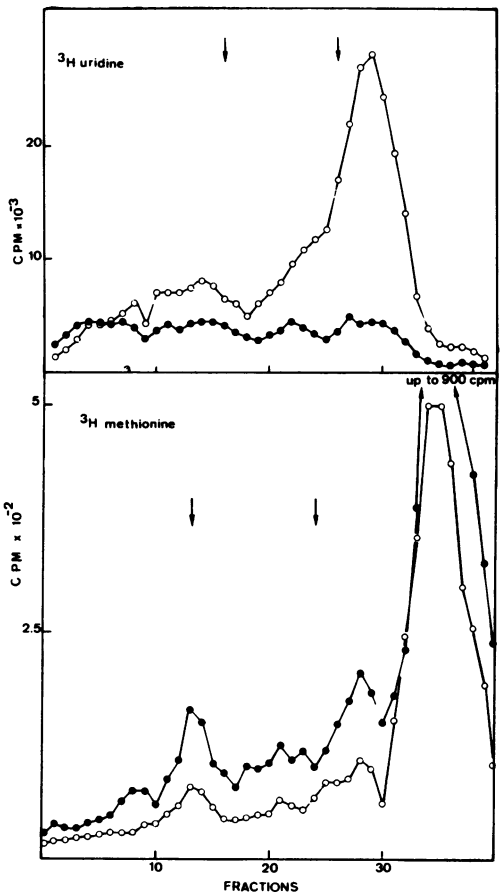

FIG. 9. *Velocity sedimentation analysis of methylated viral RNA from tsO23(III)-infected cells. Four batches of HeLa cells were infected with tsO23(III) (≈50 PFU per cell); two were incubated at 30°C, and the others were incubated at 39.2°C for 2 h in Eagle minimal essential medium with only 10^{-2} mM methionine, 2% fetal calf serum, and actinomycin D. Labels ([^{3}H]uridine or [methyl-^{3}H]methionine) were introduced at 2 h p.i. in the same medium supplemented with 10 mM sodium formate and 10 μM guanine and adenine. At 4 h p.i. the RNAs were deproteinized with proteinase K and then twice extracted with phenol-chloroform. They were analyzed on 15 to 30% sucrose gradients as usual. Symbols: ●, 30°C; ○, 39.2°C.*

made at 39.2°C were at least six times more numerous than those made at 30°C, the yield of the methyl groups they bore did not exceed that observed at 30°C. Moreover, the methylated mRNA ratio at 39.2 to 30°C was only 0.5, but this reflected only a difference in methionine metabolism between the two temperatures, as it could be seen on the figure at the level of the tRNA peaks and as we observed for the wild-type VSV (data not shown).

DISCUSSION

ts mutants of groups III and V induce at restrictive temperature a large amount of viral RNA, which determines their RNA$^+$ phenotype (10). After sucrose gradient analysis, however, an abnormal profile is observed for the RNA synthesized at 39.2°C: for both groups the relative proportion between the three main RNA species, i.e., 38, 28, and 13–15S RNA, is changed when compared with what is seen at 30°C. The pattern obtained resembles that described under other circumstances with mutants or even with the wild type (2, 17, 30) and shows also a diminution of the yield of the virion-like RNA, counterbalanced by enhancement of the RNA transcripts. Such a result has generally been explained by the existence of an equilibrium between genome replication and transcription (17, 22), which are thought to share the same template and the same enzyme(s). In the case of *ts*III and *ts*V mutants, high temperature apparently promotes upsetting of the normal equilibrium. The quantity of the 38S RNA, which is the final replication product, is reduced, more especially as the absence of budding out at 39.2°C would have lead to its expected accumulation. Further analyses here reported demonstrate that the paucity of the 38S RNA observed in a gradient profile cannot be related to a trapping of this RNA species among other viral products. Therefore, the synthesis of new genome RNA looks to be really affected when infection with *ts*III or *ts*V mutants is carried out at 39.2°C. The inability of superinfecting T particles to replicate at 39.2°C when mixed with *ts*III or *ts*V mutants sustains the idea that replication of these mutants is deficient at restrictive temperature. Kinetic curves enable us to explain more precisely what occurs at 39.2°C: there is not an arrest of the synthesis of the 38S RNA but, rather an abolishment of the normal amplification of the replication process by limitation of the number of synthesized molecules. The rate of synthesis seems to be restricted to its initial value as if, just after the entry of the parental virions, the replication round began normally at 39.2°C within the cell. An interpretation of these observations is to suppose two states of the defective polypeptide in group III and group V mutants depending on the temperature at which they have been synthesized: the alteration of the new polypeptides synthesized at 39.2°C would lead to a restriction of the synthesis of new VSV genomes, whereas the parental proteins brought by virions grown at 30°C would operate normally in this process. This latter assumption is favored by two observations: for both *ts*III and *ts*V mutants the

amount of 38S RNA at 39.2°C is dependent on input multiplicity, and in the case of group V the parental G protein can be reused in rescue of UV-irradiated virions (4).

The implication of proteins altered in complementation groups III and V in the process of VSV replication is a puzzling question. Indeed, it has been demonstrated that proteins G and M are not functional at 39.2°C in mutants of groups V and III, respectively (9, 11, 12). Our results seem to indicate that the two envelope proteins of VSV may also have a role in the intracytoplasmic synthesis of viral RNA. Such a statement may appear surprising; however, there is a good agreement between what is observed about the synthesis of 38S RNA after different temperature shifts and the reversibility (or lack of it) of the alteration induced by high temperature in polypeptide M or G for *ts*III or *ts*V mutants. Lafay and co-worker (11, 13) have demonstrated that for mutants of group III the M protein does not recover a functional state at permissive temperature when it has been synthesized at 39.2°C, whereas under the same conditions the G protein of group V mutants rapidly reverses towards normal behavior (12). In parallel, we observed that the 38S RNA did not recover normal synthesis (at least not for 1.5 h) when *ts*III-infected cells were transferred from 39.2 to 30°C; on the contrary, in *ts*-V-infected cells the return to 30°C immediately (in another experiment, not shown here, we saw that 30 min was enough) produces the recuperation of a normal level of 38S RNA synthesis. Thus, we can draw a parallel between the replication of 38S RNA and the behavior of M and G proteins. Little is known about the mechanism of new genome synthesis, but it was shown to require the nucleocapsid. Our own previous studies dealing with mutants in groups I, II, and IV (2, 3, 22), tentatively correlated with the three proteins of the capsid, have shown their implication in the replicative process. With the present knowledge, there is no reason to assign a direct role to the M and G proteins in the same process, but one may consider that these two envelope proteins have indirect intervention. Perhaps, owing to their specific ability to be inserted into the cellular membranes, they would determine some special sites in which the nucleocapsid apparatus would be devoted more to the production of genomic RNA than to that of mRNA's. The long cytoplasmic lifetime of the G protein and its specific insertion in the membrane compartment of the rough endoplasmic reticulum (8, 9, 23) as soon as it is translated are facts a priori consistent with such an hypothesis.

The second point to be discussed now also implies that the M protein might have a role other than the simple construction of the viral envelope. We have already mentioned that restriction of 38S RNA synthesis favors production of the mRNA because of the upsetting of the equilibrium between the replicating and the transcribing nucleocapsids. This phenomenon may well account for the increase of the 13-15S peak observed for mutants of group V at 39.2°C, but not for that for mutants in group III because of the striking increase of the quantity of 28 and 13-15S RNA species (up to 20 times the 30°C yield) observed as soon as *ts*III-infected cells are incubated at 39.2°C. However, this increase does not take place when primary transcription is studied at 39.2°C (personal observation). In this case, only parental proteins synthesized at permissive temperature are implicated, and, as discussed above for replication of 38S RNA, they might have preserved their expected function. The excessive synthesis of 28 and 13-15S RNAs otherwise observed at 39.2°C concerns actually the process of transcription, since these RNAs appear to be complementary to the viral genome and possess polyadenylic acid-terminal sequences. However, they cannot be characterized as polysomal RNAs: only a fraction of these RNAs is recovered as polysomes, leading nevertheless to the normal quantity of mRNA bound to ribosomes and to the same quantity of translated viral polypeptides as those during the course of an infection at 30°C. Whether this implies that the translational machinery of eucaryotic cells is fully saturated when functioning (as suggested by Laskey et al. [14]) or that viral or cellular regulation controls the quantity of protein synthesis cannot be presently resolved. It must be noticed that such regulation could occur via the degree of methylation of the mRNA's, which appeared defective for most of them. Anyway, it is clear that after the alteration of the M protein an exaggeration of the VSV transcription rate appears. This in turn suggests that the potential transcription capacity present in VSV-infected cells is not fully expressed during the course of a normal cycle and that the M protein is implicated in some way in this repression. In relation to this statement, previous results may be considered. Inhibition of the in vitro transcription activity by envelope proteins has been shown in a virus related to the rhabdovirus class (15). Uncoated nucleocapsid cores from VSV appear to be more active in the in vitro polymerase reaction than are complete VSV virions (27), and Perrault and Kingsbury specified that an inhibitor of this reaction, which is not the G protein, is present in the envelope of VSV (19). These data support our conclusion, although it might be conceivable that regulation of the transcription activity by the M protein

would not be necessarily observed in the virion but would rather be an intracytoplasmic process. This putative role for the M protein as well as the reason for the lack of translation of the excess of mRNA made at 39.2°C by *ts*III mutants, is currently under investigation.

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