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Physiological Characterization of Temperature-Sensitive Mutants of Mengovirus

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Twenty-four temperature-sensitive mutants of mengovirus were characterized physiologically with respect to phenotype. The mutants were separated into four classes on the basis of viral RNA synthesis. L-67-S cells infected with five of the mutants synthesized little viral RNA at 39.5 C. These mutants are designated RNA$^-$. One mutant is designated RNA* since its RNA synthesis is altered at both 39.5 and 31.5 C. The other mutants were divided into two groups, RNA$^\pm$ (25 to 49% of wild-type RNA synthesis) and RNA$^+$ (50 to 100% of wild-type RNA synthesis). The time of expression of the mutation in the RNA$^-$ mutants was estimated from the results of reciprocal temperature-shift experiments. The mutation in ts12 appears to be expressed at the time RNA synthesis normally begins. The defect in three of the mutants was expressed 1 to 2 h before RNA synthesis is normally detectable. Protein synthesis is required before RNA synthesis begins when the cells are shifted from 39.5 to 31.5 C. The RNA polymerase synthesized by cells infected with these RNA$^-$ mutants at 31.5 C was stable and fully active when assayed at 39.5 C in vitro. The sedimentation profiles of the viral RNA synthesized by cells infected with RNA$^+$ and RNA$^\pm$ mutants are similar to wild-type profiles with the exception of ts148. Cells infected with this RNA$^\pm$ mutant synthesize RNA that sediments in a sucrose gradient like replicative-intermediate RNA, but little mature viral RNA is evident. The results of step-up experiments indicate that the temperature-sensitive period for the majority of the RNA$^+$ and RNA$^\pm$ mutants extends through most of the replicative cycle. The temperature-sensitive defect of four of the mutants, however, was expressed in the first hour, suggesting that some undefined early function is required for the eventual maturation of mengovirus. The virions of three of the RNA$^-$ mutants were more thermolabile than wild-type virions. Five of the RNA$^+$ and RNA$^\pm$ mutants were also thermolabile. Genetic complementation at a significant level was not detectable in mixed infections of the mutants described.

Temperature-sensitive mutants of many representative groups of animal viruses have been isolated (13, 14). The unique advantage of ts mutants is that they can, in theory, be used to study any gene. Ideally, the characterization of ts mutants should couple physiological and genetic characterization so that specific functions can be linked to specific genes and gene products. This report describes the physiological characterization of 24 ts mutants of mengovirus.

MATERIALS AND METHODS

Materials. Tris, EDTA, *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), morpholinopropanesulfonic acid (MOPS), *N*-tris(hydroxymethyl)methyl-2-aminomethanesulfonic acid (TES), type 1-A RNase, cycloheximide, nucleosides, and nucleotides were obtained from Sigma Chemical Co. Sodium lauryl sulfate was obtained from Schwarz/Mann and recrystallized by the method of Schmidt (22). Permablend III was obtained from Packard Instrument Co. Ionagar-2 was obtained from Colab Laboratories, Inc. Neutral red was obtained from Fisher Scientific Corp. DEAE-dextran was obtained from Pharmacia Fine Chemicals. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (nitrosoguanidine) was obtained from Aldrich Chemical Co. [5-^{3}H]uridine and [5-^{3}H]uridine-triphosphate (tetrasodium salt) were ob- D and 5-fluorodeoxyuridine were gifts from W. B. Gall of Merck, Sharp and Dohme and W. E. Scott of Hoffman-La Roche, Inc., respectively.

Cells and virus. L-67-S cells were grown in suspension in A67 medium (C. W. Bond and H. E. Swim, submitted for publication). The basal medium

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AMB42 consists of BM42 (20) supplemented with 75 mg of succinic acid and 100 mg of sodium succinate per liter. ABM42-MTH consists of ABM42 supplemented with 10 mM each of MOPS, TES, and HEPES. A67 medium consists of 92.5% ABM42 and 7.5% calf serum (vol/vol). The wild-type (WT) mengovirus has been described previously (20).

Virus was assayed by the hemagglutination method (18) or by a modified pour-plate plaque procedure (4). Basal feeder layers containing 6 ml of A67 supplemented with 39.0 mg of Ionagar 2 and 390 μg of DEAE-dextran were prepared in 60-mm glass or plastic culture dishes. L-67-S cells were harvested by centrifugation at 600 $\times$ g for 5 min and washed twice with A67 medium. The cells were then suspended in 1 ml of the appropriate dilution of virus in ABM42-MTH at a density of 2×10^7 cells per ml. After incubation at 31.5 C for 30 min, cells were collected by centrifugation and suspended in 1 ml of 2$\times$ A67 medium. A solution containing 13 mg of Ionagar 2 and 130 μg of DEAE-dextran per ml was incubated at 50 C. The agar solution (1 ml) was mixed with the cell suspension and immediately poured on the basal feeder layer. It should be noted that variable results are obtained in this assay unless the virus is adsorbed to the cells for at least 30 min at 31.5 C prior to plating. The plates were incubated in a humidified atmosphere of 6% carbon dioxide-94% air for 48 h at 37 or 39.5 C, or for 72 h at 31.5 C. After incubation, the plates were overlaid with 2 ml of a solution containing 6.5 mg of Ionagar 2 and 0.2 mg of neutral red per ml, in phosphate-buffered saline (12). The plates were incubated for 4 h at 31.5 C, and the plaques were counted.

L-67-S cells were infected with WT or ts mutants of mengovirus in all of the experiments described as follows. L-67-S cells in the logarithmic phase of growth were harvested by centrifugation at 1,000 $\times$ g for 5 min and suspended to a density of 10^7 cells per ml of ABM42-MTH containing virus at a multiplicity of 20 PFU per cell. The suspension was incubated for 30 min with occasional mixing at the temperature indicated in the individual experiment. After incubation, the suspension was diluted to a density of 2×10^6 cells per ml with ABM42-MTH and incubated on a rotary shaker under the conditions dictated by the individual experiments. Uninfected control cells were sham treated.

Isolation of mutants. WT mengovirus was mutagenized by treating infected cells with nitrosoguanidine. L-67-S cells infected with WT and incubated at 31.5 C were supplemented with 7 μg of nitrosoguanidine per ml at 4.5 h postinfection (PI). This concentration inhibits the synthesis of viral RNA approximately 75% and the yield of infectious virus approximately 95% (unpublished data). The virus was harvested at 17 h PI. The mutagenized virus was screened for ts mutants by a replica plating procedure.

Master plates for replica plating were prepared in 100-mm culture dishes employing three times the quantities of ingredients used in the pour-plate plaque method for the 60-mm dishes. After incubation for 72 h at 31.5 C, each master plate was replica plated to two uninfected plates by the standard velveteen method described by Lederberg and Lederberg (15). One replica plate was incubated for 72 h at 31.5 C, and the second plate was incubated for 48 h at 39.5 C. The replica plates were compared for the development of plaques at 31.5 and 39.5 C. Plaques that appeared to be ts were picked from the 31.5 C plate, suspended in 1.5 ml of PBS supplemented with 5% (vol/vol) calf serum, and stored at -20 C. A neutral red overlay was not utilized in the isolation of mutants.

A spot test procedure was used to rapidly verify the ts nature of the original clone and to eliminate many of the unstable mutants which appeared in the original screen. Portions of each ts clone were spotted on duplicate uninfected pour plates with a glass rod (3-mm diameter). One plate was incubated for 72 h at 31.5 C and the duplicate for 48 h at 39.5 C. The plates were scored for the presence or absence of cell lysis.

The clones which were ts by the spot test procedure were recloned twice and then passed twice serially in L-67-S cells.

Twenty-four ts mutants were isolated by these procedures and were found to have reversion frequencies of less than 10^{-4} by the pour-plate plaque method (PFU at 39.5 C/PFU at 31.5 C).

Assays for radioactivity. Scintillation fluid II contains 730 ml of dioxane, 135 ml of toluene, 35 ml of absolute methanol, 100 g of napthalene, and 5.5 g of Permablend III. Scintillation fluid III contains 5.5 g of Permablend III in 1,000 ml of toluene.

Radiolabeled samples were prepared for assay as follows. Each sample of 0.5 ml was immediately mixed with 0.5 ml of 0.1 M sodium pyrophosphate containing 100 μg of bovine gamma globulin and 0.5 ml of 1.5 N perchloric acid. After standing in an ice bath for 15 min, the samples were diluted with 3 ml of 0.5 N trichloroacetic acid-0.06 M sodium pyrophosphate and transferred quantitatively to a filter tower fitted with a 25-mm glass-fiber filter (Whatman GF/A). The precipitate was collected and washed four times with 4 ml of 0.5 N trichloroacetic acid-0.06 M sodium pyrophosphate. After drying thoroughly, the filter was transferred to a vial, and 7.5 ml of scintillation fluid III was added.

In some experiments, radiolabeled samples were prepared for assay by the tube method described by Ward and Plagemann (24). The final precipitates were suspended in 0.1 ml of trichloroacetic acid and transferred to scintillation vials containing 15 ml of scintillation fluid II.

All samples were counted in a liquid scintillation spectrometer (Packard Tri-Carb model 2003). Counting efficiencies of 30% for ^{3}H and 84% for ^{14}C were obtained with samples prepared by either the tube or filter procedure.

Incorporation of [^{3}H]uridine into virus-specific RNA. The total virus-specific RNA synthesized by infected cells was determined as follows. The suspension of infected cells was supplemented with 0.5 μmol of 5-fluorodeoxyuridine and 1 μg of actinomycin D per ml at 30 min PI. [^{3}H]uridine was added at the concentration and specific activity dictated by individual experiments. At intervals thereafter, duplicate 0.5-ml samples were removed and assayed immedi-

ately for acid-insoluble radioactivity or frozen in a dry ice-ethanol bath and stored at −20 C for subsequent analysis.

Assay of RNA-dependent RNA polymerase. The particle-associated polymerase was prepared as described by McDonnell and Levintow (17). The activity of RNA-dependent RNA polymerase in the particulate fraction was assayed by the method of Plagemann and Swim (21).

Sedimentation analysis of virus-specific RNA. Infected cells were labeled with [^{3}H]uridine in the presence of 5-fluorodeoxyuridine and actinomycin D as described above. After labeling, the suspension was rapidly cooled in an ice bath, and the cells were collected by centrifugation at 1,000 × *g* for 10 min at 4 C and stored at −70 C. The cells were thawed and suspended in 2.5 ml of 10 mM Tris-hydrochloride (pH 7.4) and mixed with 0.25 ml of 10% sodium dodecyl sulfate (wt/vol). The mixture was then incubated for 5 min with frequent vigorous agitation and then layered on a linear sucrose gradient. The composition of the gradient and the conditions of centrifugation are described in the individual experiments.

Linear sucrose gradients were prepared with a gradient former (model 270, Instrument Specialties Co.). Gradients were fractionated in a density gradient fractionator (model D, Instrument Specialties Co.) equipped with an automatic fraction collector. The absorbance at 254 nm was monitored with a model UA-2 UV analyzer and recorded with an ISCO model 410 recorder. The fractions were analyzed in toto for acid-insoluble radioactivity.

Genetic complementation. Pairs of mutants were tested for complementation in L-67-S cells by the method of Burge and Pfefferkorn (3).

RESULTS

Mixed infections were conducted with pairs of most of the ts mutants in an attempt to separate the mutants into complementation groups (data not shown). Significant complementation was not observed as evidenced by complementation indices (3) in the range of 0.2 to 1.7.

There is considerable variation within the group of mutants with respect to heat sensitivity. For example, 35 C is a nonpermissible temperature for 11 of the 24 ts mutants (Table 1).

The mutants are also variable with regard to heat stability. The data presented in Fig. 1 indicate that the ts mutants can be assigned to three groups. The nine mutants comprising groups II and III are significantly less stable than WT, indicating alterations in structural proteins (16).

Cells infected with individual mutants were tested for their ability to synthesize virus-specific RNA. The results obtained with seven of the mutants are illustrated in Fig. 2. These curves were integrated, and the fraction of viral RNA synthesized by cells infected with ts mutants (39.5 C/31.5 C) relative to WT was calculated. These data, which are summarized for all of the mutants in Table 1, demonstrate that the mutants can be assigned to four groups with respect to viral RNA synthesis. Mutants which permit a normalized fraction of viral RNA synthesis of 50 to 100% of WT are designated RNA$^+$. Those permitting 25 to 49% of WT are designated RNA$^\pm$. The ts mutants which permit less than 25% of WT synthesis are

TABLE 1. *Summary of some of the phenotypic characteristics of ts mutants of mengovirus*

Virus	RNA synthesis[a]	Permissivity at 35 C[b]	End of ts period (h PI)[c]
WT	1.00 +	+	none
ts4	0.76 *	+	13
ts12	0.22 −	−	7
ts25	0.50 +	+	2.5
ts26	0.13 −	+	7
ts36	0.83 +	−	9
ts37	0.76 +	−	7
ts46	0.77 +	+	1
ts51	0.76 +	+	1
ts74	0.41 ±	−	5.5
ts79	1.01 +	−	8.5
ts82	0.59 +	+	1
ts88	0.11 −	−	9
ts95	0.05 −	+	8
ts100	1.01 +	+	9
ts105	0.05 −	−	8.5
ts111	0.83 +	−	8.5
ts115	0.69 +	+	8.5
ts116	0.46 ±	+	4
ts130	0.53 +	+	1
ts143	0.26 ±	−	8.5
ts148	0.38 ±	−	8.5
ts186	0.46 ±	+	8.5
ts189	0.62 ±	−	8.5
ts190	0.32 ±	+	8.5

[a] The fraction of RNA synthesized by cells infected with ts mutants (39.5 C/31.5 C) was determined by integration of the data obtained in the experiment described in the legend to Fig. 2. The fraction was then normalized to a WT value of 1.00. A fraction of RNA synthesized equal to 0.00 to 0.24 is indicated by (−), 0.25 to 0.49 by (±), and 0.50 to 1.00 by (+). The symbol (*) indicates altered RNA synthesis at both 39.5 and 31.5 C.

[b] Mutants were tested for temperature sensitivity by the pour-plate plaque assay at 35.0 as well as 31.5 and 39.5 C. +, Permissive at 35 C; −, nonpermissive at 35 C.

[c] The end of the ts period was estimated in temperature step-up experiments (Fig. 5). The estimated end of the ts period is the step-up time in which the virus yield at 18 h PI was greater than 25% of the WT yield at 39.5 C.

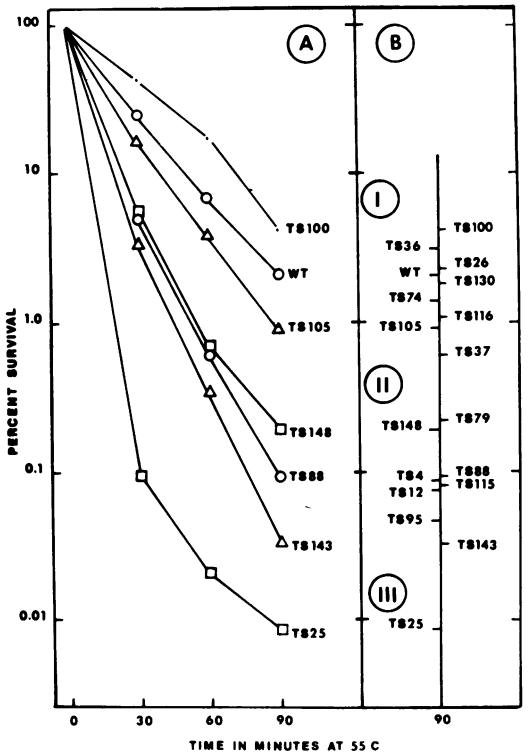

FIG. 1. *The kinetics of heat inactivation of WT and ts mutants at 55 C. Virus stocks were diluted fivefold into phosphate-buffered saline containing 5% calf serum (vol/vol) and heated at 55 C in a constant-temperature water bath for the indicated time, and the surviving virus was assayed with L-67-S cells by the pour-plate plaque method at 31.5 C.*

designated RNA$^-$. Mutant ts4 is designated RNA* because it is defective in RNA synthesis at both 31.5 and 39.5 C. Synthesis of viral RNA by cells infected with ts4 begins about 1 h later than cells infected with WT and proceeds at a reduced rate.

The RNA synthesized by cells infected with the RNA$^+$ and RNA$^\pm$ mutants was subjected to sedimentation analysis on sucrose gradients. The sedimentation profiles for representative mutants are shown in Fig. 3. Viral RNA from cells infected with WT sediments from 18*S* to 40*S* with a prominent peak at 35*S*. The 35*S* peak contains single-stranded viral RNA and the replicative intermediate (RI-RNA) sediments over the range of 18*S* to 40*S* (1). Many of the mutants exhibited a WT profile as exemplified by ts190 (Fig. 3). With others, the RNA synthesized is more heterogeneous than WT, as exemplified by the profile for ts37. Mutant ts148 is unique to this series in that most of the RNA synthesized is heterogeneous and sediments from 18*S* to 40*S* without a prominent peak at 35*S* (Fig. 3). The results of other studies with several of the RNA$^+$ and RNA$^\pm$ mutants indicate that single-stranded RNA is not incorporated into particles of sufficient maturity to protect it from RNase (19) at the nonpermissive temperature (data not shown).

It was of interest to ascertain whether the RNA polymerase synthesized by cells infected with RNA$^-$ mutants at 31.5 C is thermolabile. The data presented in Table 2 illustrate that the particulate RNA-dependent RNA polymerase synthesized at 31.5 C by cells infected with ts12 and ts88 functions as well in vitro at 39.5 C as that from cells infected with WT. These data suggest that cells infected with RNA$^-$ mutants

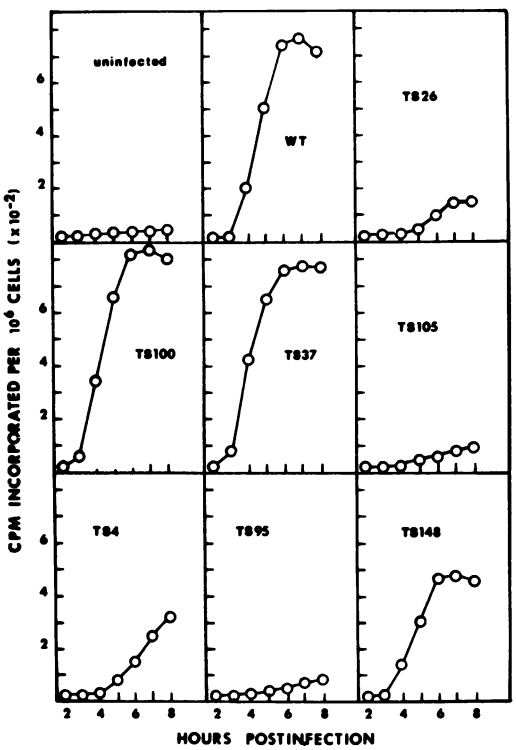

FIG. 2. *RNA synthesis by cells infected with virus at 39.5 C. L-67-S cells were infected with 20 PFU of virus per cell at 31.5 C as described in Materials and Methods. At 5 min PI, one-half of the suspension was incubated at 39.5 C, and the other half was incubated at 31.5 C (data not shown). The suspensions were supplemented with 1 μg of actinomycin D and 0.5 μmol of 5-fluorodeoxyuridine per ml at 30 min PI. At 2 h PI, the suspensions were supplemented with [^{3}H]uridine (10 μCi/μmol, 0.025 μmol/ml). Duplicate samples were removed at intervals thereafter and the counts per minute of [^{3}H]uridine incorporated into acid-insoluble material were determined by the tube method.*

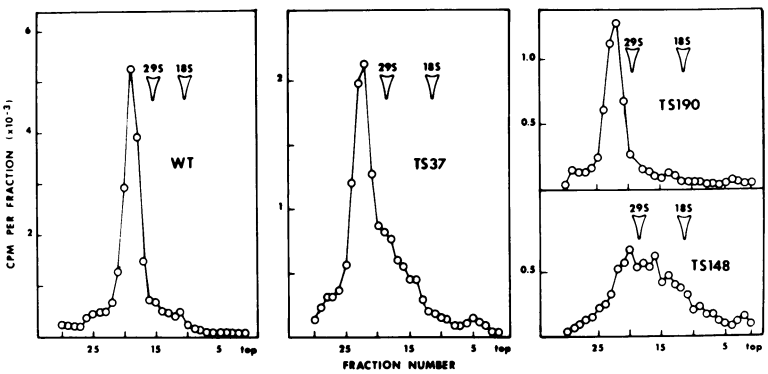

FIG. 3. *Sedimentation analysis of viral RNA synthesized by L-67-S cells infected with WT or ts mutants. Cells were infected with 20 PFU of virus per cell at 31.5 C. At 20 min PI, the incubation temperature was increased to 39.5 C. At 3 h PI, the medium was supplemented with 0.50 μmol of fluorodeoxyuridine and 1 μg of actinomycin D per ml. At 3.25 h PI, the medium was supplemented with [³H]uridine (50 μCi/μmol, 0.025 μmol/ml). The cells were harvested at 4 h PI by centrifugation at 1,000 × g for 10 min at 4 C, and the pellets were stored at −70 C. The cell pellet was subsequently thawed, lysed, and analyzed on sucrose gradients. The conditions of centrifugation were: 0.15 to 0.9 M sucrose in 50 mM NaCl, 10 mM EDTA, 10 mM Tris-hydrochloride (pH 7.4); SW25.1 rotor, 11 h, 25,000 rpm, 20 C. The gradients were fractionated and monitored for absorbance at 256 nm, and each fraction was assayed for acid-insoluble material by the tube method. The arrows indicate the position of the 18S and 29S ribosomal RNA as estimated from the absorbance profile.*

TABLE 2. *Comparison of RNA-dependent RNA polymerase activity of postnuclear fractions from cells infected with WT or RNA⁻ mutants*

Virus	Incubation temperature of infected cells (C)	Counts/min incorporated per 10⁷ cells[a]	
		Assay temperature	
		31.5 C	39.5 C
WT	31.5	3,277	4,295
	39.5	1,829	1,771
ts12	31.5	2,834	3,216
	39.5	231	219
ts88	31.5	2,552	3,496
	39.5	273	287
Uninfected	31.5	192	237
	39.5	248	222

[a] L-67-S cells were infected with a multiplicity of 20 PFU per cell of WT, ts12, or ts88 at 31.5 C. At 20 min PI, the suspensions were divided into 2 equal portions, and the incubation temperature of one was increased to 39.5 C. The second portion was maintained at 31.5 C. At 4.5 and 9 h, respectively, the cells were harvested, and postnuclear fractions were prepared and assayed at 31.5 and 39.5 C.

either do not synthesize RNA polymerase at 39.5 C or the polymerase synthesized is inactive or degraded.

As a prerequisite to studies of the temporal relations of the ts defects, it was necessary to compose the time sequence of viral development at 31.5 and 39.5 C. The data presented in Fig. 4 illustrate that synthesis of viral RNA and the production of virus begin 2.5 to 3 h earlier at 39.5 C than at 31.5 C. It should also be noted that more RNA and virus are produced at 31.5 than at 39.5 C.

The time of expression of mutant defects was estimated in temperature step-up experiments. If the ts defect is fully expressed before the shift from permissive to nonpermissive temperature, a normal yield of virus should be expected. Similarly, if the defect is not expressed before the temperature shift, a reduced yield of virus should be obtained. If the defect is expressed over a prolonged period, a gradual increase in yield as a function of step-up time should be evident. The results of several temperature step-up experiments employing representative mutants are shown in Fig. 5. The viral yield was determined by the hemagglutination procedure. The hemagglutination activity of suspensions of WT mengovirus and each of the 24 ts mutants is proportional to the infectivity as measured by the pour-plate plaque assay. The apparent increase in viral yield when the temperature is stepped up with WT and ts46 at 8 h or later reflects the fact that the yield of virus is greater at 31.5 than at 39.5 C (Fig. 4). The ts defect in ts46 is expressed in the first hour, whereas the defects in the other mutants shown in Fig. 5 are operative over most of the replicative cycle. Similar experiments were conducted with the other mutants not shown in Fig. 5. These curves were used to establish the ts period before 25% of the WT yield of virus is obtained. The overall data which define the length of the ts period in

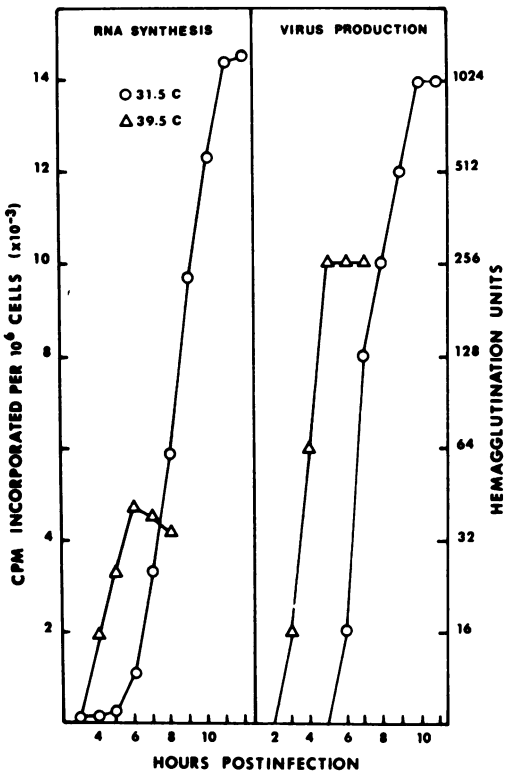

FIG. 4. *Temporal relation of viral-specific RNA synthesis and virus production. L-67-S cells were infected with 20 PFU of WT per cell at 31.5 C. At 5 min PI, the suspension was divided, and the incubation temperature of one portion was increased to 39.5 C. The second portion was maintained at 31.5 C. At 45 min PI, the incubation media were supplemented with 1 μg of actinomycin D and 0.50 μmol of 5-fluorodeoxyuridine per ml. At 3 h PI, the incubation media were supplemented with [³H]uridine (25 μCi/μmol, 0.025 μmol/ml). At intervals thereafter, duplicate 0.5-ml samples were removed and the counts per minute incorporated into acid-insoluble material were determined by the filter method. At intervals throughout the replicative cycle, samples were removed and assayed for hemagglutinating activity.*

the 24 mutants are summarized in Table 1.

Temperature step-down experiments were employed to examine in more detail the time of expression of the defects in the RNA⁻ mutants. The lag period from the time of step-down to the beginning of viral RNA synthesis provides an estimate of the period from the earliest expression of the mutation to the time that RNA synthesis would normally begin in cells infected with WT. The results of a typical step-down experiment are shown in Fig. 6. The data indicate that cells infected with WT are capable of synthesizing RNA at a maximal rate when the temperature is stepped down from 39.5 to 31.5 C at 4 h PI. Cells infected with ts105, on the other hand, do not synthesize RNA maximally after step-down until after a lag period of about 2 h. Similar experiments were conducted with ts105 and the other RNA⁻ mutants in which the temperature was stepped down at 3, 4, and 5 h PI. The data obtained was analyzed by the least-squares method to determine the linear portion of the step-down RNA-synthesis curve. The abscissa intercepts of these lines were calculated from the equations of the lines. The data for cells infected with ts mutants were normalized to those for WT by superimposing the 31.5 C controls at the abscissa. The normalized data were used to calculate the lag period between step-down and the synthesis of RNA at a linear rate (Table 3). The defects in ts26, ts88, and ts105 appear to be expressed about 1 to 2 h before RNA synthesis would occur at a maximal rate in cells infected with WT. The data for ts12, on the other hand, indicate that the mutation is expressed at about the same time as RNA synthesis would normally occur.

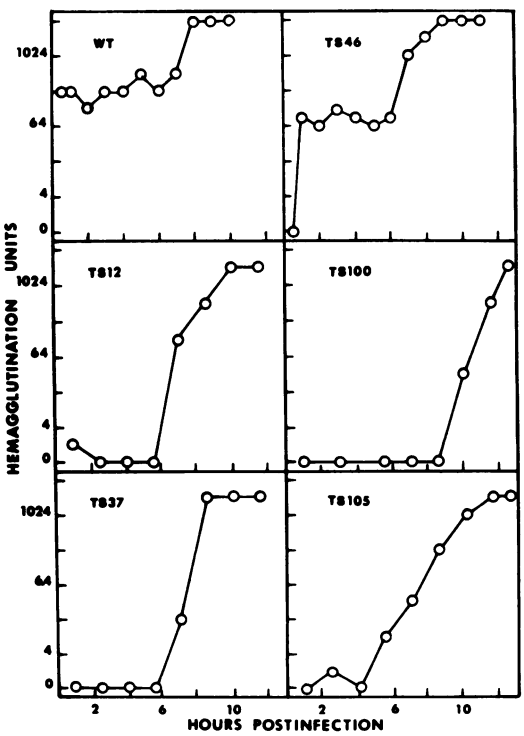

FIG. 5. *Effect of nonpermissive temperature on the yield of virus as a function of temperature-shift time after infection. L-67-S cells were infected with 20 PFU of virus per cell at 31.5 C. At intervals thereafter, portions were removed and incubated at 39.5 C. At 18 h PI, the suspensions were stored at −20 C and later assayed for the yield of virus by the hemagglutination procedure.*

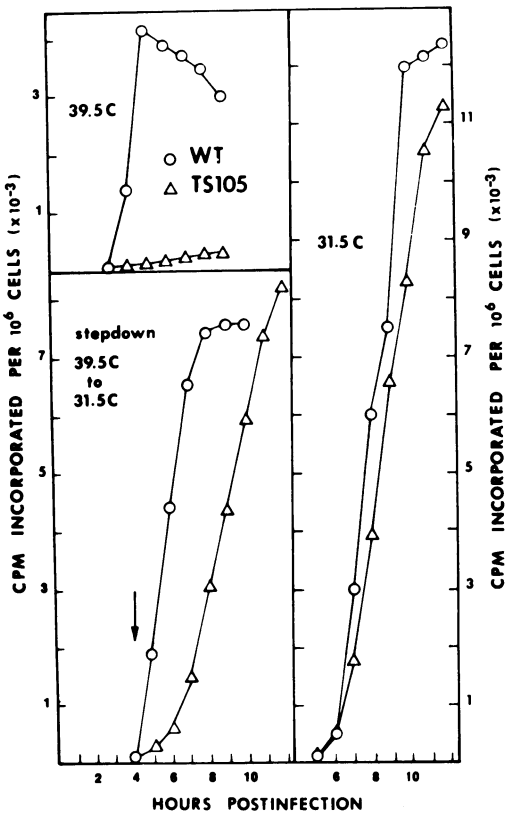

FIG. 6. *The effect of stepping down the temperature from 39.5 to 31.5 C on viral-specific RNA synthesis by cells infected with WT or ts105. L-67-S cells were infected with 20 PFU of virus per cell at 31.5 C. At 5 min PI, the suspension was divided, and the incubation temperature of one portion was increased to 39.5 C. The second portion was maintained at 31.5 C. At 35 min PI, the media were supplemented with 1 μg of actinomycin D and 0.50 μmol of 5-fluorodeoxyuridine per ml. At 4 h PI, a portion of the suspension incubated at 39.5 C was shifted to 31.5 C and supplemented with [³H]uridine (10 μCi/μmol, 0.025 μmol/ml). Control suspensions maintained at 39.5 and 31.5 C were supplemented with [³H]uridine (10 μCi/μmol, 0.025 μmol/ml) at 3 and 5 h PI, respectively. Duplicate 0.5-ml samples were removed at intervals thereafter and assayed for the counts per minute incorporated into acid-insoluble material by the filter method.*

It was of interest to ascertain whether synthesis of protein is required for RNA synthesis after step-down in view of the lag observed with three of the mutants. The data presented in Fig. 7 demonstrate that RNA synthesis does not occur after step-down in the presence of cycloheximide. These data indicate that protein synthesis is required.

TABLE 3. *The length of the lag period after step-down to permissive temperature before linear RNA synthesis begins in infected cells as a function of time of stepdown*

Virus	Length of lag period after step-down (h)[a]		
	3 h Step-down	4 h Step-down	5 h Step-down
WT	0.3	0.1	0.1
ts12	0.8	0.0	0.0
ts26	2.0	1.2	0.8
ts88	1.8	1.0	0.8
ts95	1.8	0.9	0.7
ts105	2.2	1.5	1.2

[a] The length of the lag period was calculated from the time of step-down to the beginning time of linear RNA synthesis after step-down from 39.5 to 31.5 C.

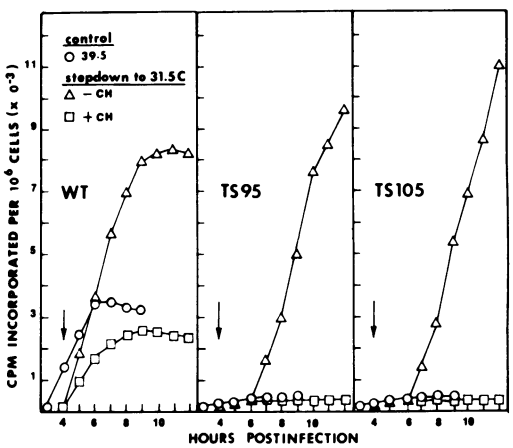

FIG. 7. *Dependence of viral-specific RNA synthesis on protein synthesis after a temperature-shift from 39.5 to 31.5 C. L-67-S cells were infected with 20 PFU of virus per cell at 31.5 C. At 5 min PI, the suspensions were divided into four portions. The incubation temperature of three portions was increased to 39.5 C, and the fourth portion was maintained at 31.5 C. At 45 min PI, the medium was supplemented with 0.50 μmol of 5-fluorodeoxyuridine and 1 μg of actinomycin D per ml. At 4 h PI, the incubation temperature of two of the portions maintained at 39.5 C was decreased to 31.5 C (one portion in the presence of 20 μg of cycloheximide per ml and the second in the absence of cycloheximide). The cell suspensions were supplemented with [³H]uridine (10 μCi/μmol, 0.025 μmol/ml) as follows: 39.5 C control at 3 h PI; step-down plus cycloheximide at 4 h PI; step-down minus cycloheximide at 4 h PI; and 31.5 C control at 5 h PI. Duplicate samples were removed at intervals thereafter and assayed for counts per minute incorporated into acid-insoluble material by the filter method.*

DISCUSSION

The 24 ts mutants of mengovirus described in this study collectively display a variety of phenotypes. In spite of this, the mutants do not complement each other in mixed infections. Similar results were obtained with ts mutants of poliovirus (5). The lack of complementation could result from a defect due to a lability of the mutant template at 39.5 C. However, the results of recent studies indicate that many of the ts mutants are defective in post-translational cleavage of precursor polypeptides (C. W. Bond and H. E. Swim, submitted for publication). These results may explain the failure of the ts mutants of mengovirus to complement each other in mixed infections.

The ts mutants are divided into four classes on the basis of the relative amount of viral-specific RNA synthesized by infected cells at 39.5 C (Fig. 2, Table 1). Mutant ts12 (Table 1) is included in the RNA^- group because cells infected with it produce less than 25% as much viral RNA as cells infected with WT. Cells infected with the other RNA^- mutants do not synthesize significant amounts of viral RNA. The polymerase synthesized at 31.5 C by cells infected with two of the RNA^- mutants is equally active in vitro at 31.5 and 39.5 C (Table 2). Further, the results of the experiments presented in Fig. 7 and Table 3 demonstrate that there is a lag period before RNA synthesis begins when cells infected with RNA^- mutants are stepped down from 39.5 to 31.5 C. The data presented in Fig. 7 demonstrate that the de novo synthesis of protein is required for the production of polymerase after step-down from 39.5 to 31.5 C. The results of these experiments indicate that if a product of the polymerase gene is synthesized at 39.5 C, it is either inactive or degraded at the nonpermissive temperature. Alternatively, the defect in the RNA^- mutants may be expressed in some unspecified step preceding the production of active polymerase.

Cooper and co-workers (9–11) have described a class of ts mutants of poliovirus which is similar to ts148 since the RNA synthesized sediments in a sucrose gradient like RI-RNA. Cells infected with a second class of RNA^- mutants of poliovirus do not synthesize significant amounts of viral RNA. It was concluded from physiological and genetic data (6–8) that the polymerase consists of two distinct enzymatic activities, one to synthesize the RF-RNA and a second to produce single-stranded RNA via the RI-RNA. The data presented in this report with respect to ts148 can be interpreted in an alternative way. The expression of this mutation results in the synthesis of RNA that sediments like RI-RNA, but mature single-stranded RNA is not evident. This would suggest that a second activity, possibly a regulatory protein, is required for either the completion of the mature viral RNA or for its release from the RI-RNA. The virions of ts148 are also thermolabile (Fig. 1), which indicates that a structural protein may be involved in the synthesis of mature viral RNA. Cooper et al. (11) have described a ts mutant of poliovirus with a phenotype like ts148 and have suggested that a defective structural protein has a suppressive effect on RNA synthesis. However, it cannot be ruled out from these data that the thermolability of ts148 could be due to a mutation at a second site in the genome.

The results of the step-up experiments presented in Fig. 3 and summarized in Table 1 illustrate the wide variation in the ts period of the mutants in the RNA^+ and $RNA^\pm$ classes. The ts period for cells infected with several of the mutants appears to extend through most, if not all, of the replicative cycle. The end of the ts period cannot be estimated with any degree of precision for these mutants because the synthesis of virus at 31.5 C begins to obscure the release of the mutant defect after 6 or 7 h (Fig. 4 and 5). On the other hand, the ts defect of four of the mutants is expressed in the first hour. Uncoating must occur normally, since these mutants are RNA^+. The data suggest that some undefined function that is expressed in the first hour is required for the eventual maturation of mengovirus. Similarly, ts25 has a ts period of only 2.5 h. This mutant is of additional interest because it is thermolabile (Fig. 1).

The sedimentation profiles of RNA synthesized by cells infected with the RNA^+ and $RNA^\pm$ mutants are similar to WT (Fig. 3). The amount of RNA synthesized at 39.5 C by cells infected with these mutants varies from 26 to 100% of that of WT (Table 1). Reductions in the amount of viral RNA synthesized by cells infected with ts mutants have been observed with poliovirus (11), Sindbis virus (2), and with Semliki forest virus (23). A specific explanation for this reduction in synthesis is not available.

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