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RNA Synthesis in Temperature-Sensitive Mutants of Vesicular Stomatitis Virus

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T-particle-free stocks of temperature-sensitive mutants representing the four Glasgow complementation groups of the Indiana serotype of vesicular stomatitis virus were used to study RNA synthesis at the permissive and nonpermissive temperatures of 31 and 39 C, respectively. Mutants selected from the four Glasgow complementation groups were characterized on the basis of particle and ribonucleoprotein formation. Intracellular RNAs were further characterized by polyacrylamide gel electrophoresis. *ts* G22 (group II) and *ts* G41 (group IV), previously characterized as RNA negative at the nonpermissive temperature, synthesized low levels of RNA which could not be attributed to contaminating levels of revertants. Furthermore, the levels of synthesis could not be reduced by the addition of cycloheximide. These data suggest that *ts* G22 (group II) and *ts* G41 (group IV) contain a thermally stable, virion-encapsidated transcriptase, but fail to amplify RNA synthesis due to a thermally labile function presumably necessary for the synthesis of viral RNA. *ts* G31, a group III mutant, synthesized intracellular RNA at amplified levels at the nonpermissive temperature. Intracellular ribonucleoprotein complexes were isolated in copious amounts; however, no particles corresponding in size to finished virions were observed. These data suggest a thermally labile maturation factor or envelope associated structural protein to be defective in *ts* G31 (group III). *ts* G11 (group 1) showed no detectable RNA synthesis at the nonpermissive temperature. These data suggest *ts* G11 (group I) contains a thermally labile component involved in early transcription. This group may contain a number of mutants defective in different components of the transcription apparatus, which may not complement in vivo because of the physical improbability of subunit exchange between virion particles of the incoming inoculum.

Several investigators have reported the isolation of spontaneously arising (4, 5) or chemically derived (7, 16) temperature-sensitive mutants of vesicular stomatitis virus (VSV). Pringle originally isolated four complementing groups of temperature-sensitive mutants from the Indiana serotype of VSV. Groups I and IV were classified as being RNA negative (RNA-) at the nonpermissive temperature, group III was classified as RNA positive (RNA+), whereas group II contained two members, one RNA+ and one RNA- (3). Holloway and Flamand independently reported the existence of a fifth complementation group not found in the Glasgow collection (D. V. Cormack, A. F. Holloway, and C. R. Pringle, submitted for publication; reference 6). Recent collaborative projects between Pringle and Flamand (6) and Pringle and Holloway (D. V. Cormack, A. F. Holloway, and C. R. Pringle, submitted for publication) have established the homologies of their mutants by cross-complementation. With the demonstration of a virion encapsidated transcriptase in VSV (1), and characterization of the in vivo activity of the enzyme in the absence of protein synthesis (9, 12, 14), the existence of three complementing RNA- groups in the Glasgow collection has led to a molecular conundrum. To envision complementation by two RNA- mutants (negative in transcription and replication), it becomes necessary to postulate the physical exchange of protein subunits. Although this mechanism is possible, it is unlikely that the complementation efficiencies would be as high as those observed with the input multiplicities of infection used. It seems likely that low levels of transcriptional synthesis would be necessary to account for the observed complementation levels taking place in these mutants.

Recent experiments by Wong, Holloway and Cormack (23) indicate the synthesis of low levels of acid precipitable RNA at the nonpermissive temperature in mutants homologous to Glasgow group IV.

Printz-Ane, Combard, and Martinet (18) showed that *ts* 0100 (homologous to Glasgow group IV) synthesized RNA corresponding in size to the 18 to 13*S* message fraction; the authors, however, were unable to detect synthesis of ~30*S* message. In view of these findings, it seemed desirable to reexamine the Glasgow mutants at the nonpermissive temperature at high multiplicities of infection. These conditions allow detection of low level transcription. The results of such studies are presented in this communication.

MATERIALS AND METHODS

Cells. BHK-21 C13 cells were used throughout. Cells were grown to confluency in 32 oz (907.20 g) prescription bottles (Kerr Glass Manufacturing Company Lancaster, Pa.). GIBCO BHK-21 medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% calf serum and 10% tryptose phosphate in an atmosphere of ~5% CO_2 was used during cell growth. Cells were incubated in Hythermco incubators (Pennsauken, N. J.) adjusted to 37 C.

Viral stocks. The conditions of viral infections were similar to those described with the following exception. During viral infections GIBCO BHK-21 medium supplemented with 10% calf serum in an atmosphere of ~5% CO_2 was used. Cells were infected for 15 min in incubators (described in previous paragraph) adjusted to 31 C prior to addition of prewarmed medium. T-free B-particle stocks were prepared by a modification of the procedure described by Stampfer et al. (22). Isolated plaques containing ~10^4 PFU were enriched in 32 oz prescription bottles containing ~6×10^7 cells/bottle. Although it is possible that genetic variants were selected by cloning, upon undiluted passage the isolates selected produced the same T particle as the original Pringle isolates (19). Infections were allowed to proceed at 31 C until cytopathic effects became evident. Supernatants were combined, and virus particles were pelleted in a Beckman R-30 rotor, at 23,000 rpm and 4 C for 2 h. Viral pellets were suspended overnight at 4 C in 3E buffer (0.12 M Tris-acetate; 0.06 M sodium acetate; 0.003 M EDTA, pH 7.4) and centrifuged over a 25-ml linear 5 to 40% sucrose gradients in 3E buffer in a Beckman SW 25.1 rotor at 22,000 rpm and 4 C for 90 min. Viral zones were removed with a Pasteur pipette and dialyzed overnight at 4 C against 3E buffer to remove sucrose. The dialyzed viral zones were diluted 10-fold and divided into 1- to 1.5-ml samples and stored at −80 C until used. Viral stocks were titered for infectivity at 31 and 39 C by standard plaque assay. All viral stocks used in this study contained between 5×10^9 to 5×10^{10} PFU/ml at 31 C, and showed a reduction in plaque forming ability at 39 C of ~5 to 6 $\log_{10}$.

Virus stocks were purified to remove possible contamination with cores, core aggregates, and virion aggregates. This procedure was also used to insure that no visible T zones had been generated during enrichment. Attempts to demonstrate the presence of T particles by radioactivity also proved negative as described later in the text.

Viral infections. 100-mm Petri dishes or 16 oz (453.6 g) prescription bottles both containing ~3×10^7 cells upon confluency were used throughout these experiments. Confluent monolayers were infected with 50 PFU/cell. After a 15-min adsorption period at the appropriate temperature, 5 ml of prewarmed medium containing 10 μg of actinomycin D per ml (a gift from Merck, Rahway, N. J.), 20 μCi of [5, 6-^3H]uridine per ml (New England Nuclear Corp.; specific activity 50 Ci/mol), and 50 μg of cycloheximide per ml (Parke, Davis and Co., Detroit. Mich.), where appropriate, were added to each plate. Infections were allowed to proceed for 6 h at either 31 or 39 C before intracellular RNA species were extracted. In studies involving production of viral particles or viral ribonucleoprotein the infection was allowed to proceed for the first 5 h in medium without radioactive label containing 10 μg of actinomycin D per ml as described above. At the end of 5 h, the nonradioactive medium was removed, and prewarmed medium containing 20 μCi of ^{3}H-uridine per ml and 10 μg of actinomycin D per ml was added. The infection was allowed to proceed from 5 to 8 h in the presence of radioactive label. At the end of 8 h, the entire sample was frozen at −20 C overnight to disrupt cells.

Preparation of intracellular RNA. Intracellular RNA was prepared by a modification of the procedure of Sherrer (20). It should be noted that little quantitative or qualitative differences could be obtained in RNA profiles, whether RNA extractions were performed at pH 7, 7.4, 8, or 9 in the presence of 2-mercaptoethanol or at various ionic strengths.

Intracellular RNAs were extracted after 6 h of incubation; a lengthy incubation time was used to incorporate large numbers of counts and to insure detection of any possible low level synthesis of 45*S* RNA.

After 6 h of infection, radioactive medium was removed, and the monolayers were washed with E buffer. Fresh E buffer was added, and the samples were adjusted to 1% (wt/vol) in sodium dodecyl sulfate An equal volume of 88% (vol/vol) water saturated phenol, 12% (vol/vol) m-cresol containing 0.9 g of 8-hydroxy quinolinol per liter, preheated to 60 C, was added directly to the plate.

After thorough mixing, samples were removed and placed in 30-ml Corex tubes. Samples were vigorously vortexed before heating for 2 min in a 60 C water bath. The phases were separated in a Sorvall rotor SS 34 at 9,000 rpm for 10 min at 4 C. The aqueous phase was removed and placed in fresh phenol-cresol. The phenol phase was removed and discarded. The remaining protein interface was suspended in fresh buffer and phenol-cresol, vortexed, incubated at 60 C, and the phases were separated. The resultant aqueous phase was combined with the aqueous phase obtained from the first extraction. The combined phases were extracted with phenol-cresol as before. The resultant

nucleic acids were twice precipitated in two volumes of absolute ethanol at −20 C. After the second ethanol precipitation, samples were suspended in 5 ml of DNase buffer (0.01 M Tris-hydrochloride; 0.01 M NaCl; 0.001 M $MgCl_2$, pH 7.1), to which ^{14}C labeled BHK-21 cell ribosomal RNA markers were added. The samples were adjusted to 10 μg of DNase per ml (RNase-free, Worthington Biochemical, Freehold, N.J.) and incubated in an ice bath for 1 h. After DNase treatment, the samples were precipitated in two volumes of absolute ethanol. Samples were suspended in 0.3 ml of distilled water and solid RNase-free sucrose (Schwarz Bioresearch, Inc., Orangeburg, N.Y.) was added to ~15% (wt/vol), prior to polyacrylamide gel electrophoresis. The production of ^{14}C RNA markers was by the same procedure without DNase treatment. During initial experiments when optimal conditions for extractions were sought, ^{14}C ribosomal RNA markers were present throughout the extraction procedure. No degradation of markers were observed when extractions were performed in this manner.

Preparation of virus particles and ribonucleoprotein. Infected monolayers designed to study virus and ribonucleoprotein (RNP) formation were frozen overnight at −20 C. After thawing samples were pelleted in a Beckman SW 40 rotor at 30,000 rpm and 4 C for 120 min. The supernatant fluid was removed, and the pellets were suspended overnight at 4 C in 0.5 ml of 3E buffer. Any remaining cell debris was removed by low speed centrifugation in a Sorvall GLC-1 desk top centrifuge at 200 × *g* for 2 min. The supernatant was removed and loaded on a 4.8-ml linear 5 to 40% sucrose gradient in 3E buffer. Samples were centrifuged in a Beckman SW 50.1 rotor at 30,000 rpm and 4 C for 60 min. Four-drop fractions were collected by bottom puncture onto 2.4-cm glass fiber filters (Reeve Angel). After drying, samples were counted in 10 ml of a toluene scintillation fluid containing 4 g of 2,5-diphenyloxazole (PPO) per liter + 0.05 g of 1,4-bis-2-(5 phenyloxazolyl)-benzene per liter with a Beckman LS-250 liquid scintillation spectrometer.

Polyacrylamide gel electrophoresis. The electrophoresis of RNA was performed as described by Bishop, Claybrook and Spiegelman (2). The entire intracellular RNA sample, obtained as described above, containing ^{14}C labeled BHK-21 ribosomal RNA markers was loaded on the gel. Samples were subjected to electrophoresis for 180 min at 10 mA/gel. The gels were frozen in dry ice and cut into 1-mm slices with a Mickle Gel Slicer (Brinkman Instrument, Inc., Westbury, N.Y.) and placed on Whatman filter paper strips. All slices were burned in a Packard Tri-Carb Sample oxidizer Model 305. This procedure enables the separation of ^3H counts from ^{14}C counts in the form of ^3H_2O and $^{14}CO_2$, thus enabling high efficiency of ^3H determination with no ^{14}C spillover. ^{14}C samples were counted in 15 ml of scintillation fluid containing 14.2 g of PPO and 0.94 g of *p*-bis-(*O*-methylstyryl-benzene) (bis-MSB; Packard Instrument Co., Downers Grove, Ill.) in 1 liter of toluene. ^3H samples were counted in 10 ml of scintillation fluid containing 100 g of naphthalene, 5 g of PPO, 0.6 g of 1,4-bis-2-(5 phenyloxazolyl)-benzene, 70 ml of methanol, and 250 ml of toluene made up to one liter in 1,4-dioxane.

RESULTS

Sucrose gradient analysis of viral particles and RNP cores. The temperature-sensitive mutants used in this study were *ts* G11, *ts* G22, *ts* G31, and *ts* G41, representing groups I, II, III, IV, respectively (15). Figure 1 represents the sucrose gradient profiles of RNA in subviral and viral particles obtained from infections with the four mutants at 31 and 39 C. The absence of radioactive peaks corresponding to the position of defective articles should be noted. *ts* G11 (group I) and *ts* G22 (group II) produce a characteristically long defective particle which would peak at fraction 21 to 22 under these conditions of sedimentation. *ts* G41 (group IV) and *ts* G31 (group III) characteristically produce shorter defective particles which would appear as peaks of radioactivity in fractions 25 to 26 and 29 to 30, respectively (J. T. Unger and M. E. Reichmann, Abst. Annu. Meeting Amer. Soc. Microbiol., Philadelphia, p. 195, 1972; reference 19).

Figure 1C shows the sucrose gradient profile of RNA in subviral and viral particles obtained from infection with *ts* G31 (group III) at 31 and 39 C. B particles and 140*S* RNP cores are clearly identifiable when grown at 31 C. At 39 C, however, no detectable B-particle zone is seen, although large amounts of RNP core are observed. When these cores were collected from sucrose gradients and the RNA was isolated and characterized by polyacrylamide gel electrophoresis, the predominant RNA species observed was 45*S* with minor components spreading into the 28*S* region (Fig. 2). 45*S* RNA was also observed in the core fraction obtained at 31 C (not shown). Similar results have been observed by other investigators (8, 10, 13, 21). The two fraction displacement of the core obtained at 31 C is more than likely due to variations in the technique, or increased sample size, or both. The accumulation of RNP cores in the cytoplasm of cells infected with *ts* G31 (group III) at the nonpermissive temperature is entirely consistent with temperature shift experiments of Duncan and Pringle (3), which indicate that a thermally labile function is needed late in infection. Attempts to identify the abnormal structural protein in this group have led to discrepant results (11, 17, 24), and the exact nature of the structural block remains to be elucidated.

Figure 1A, B, and D shows the sucrose gradient profile of *ts* G11 (group I), *ts* G22 (group II),

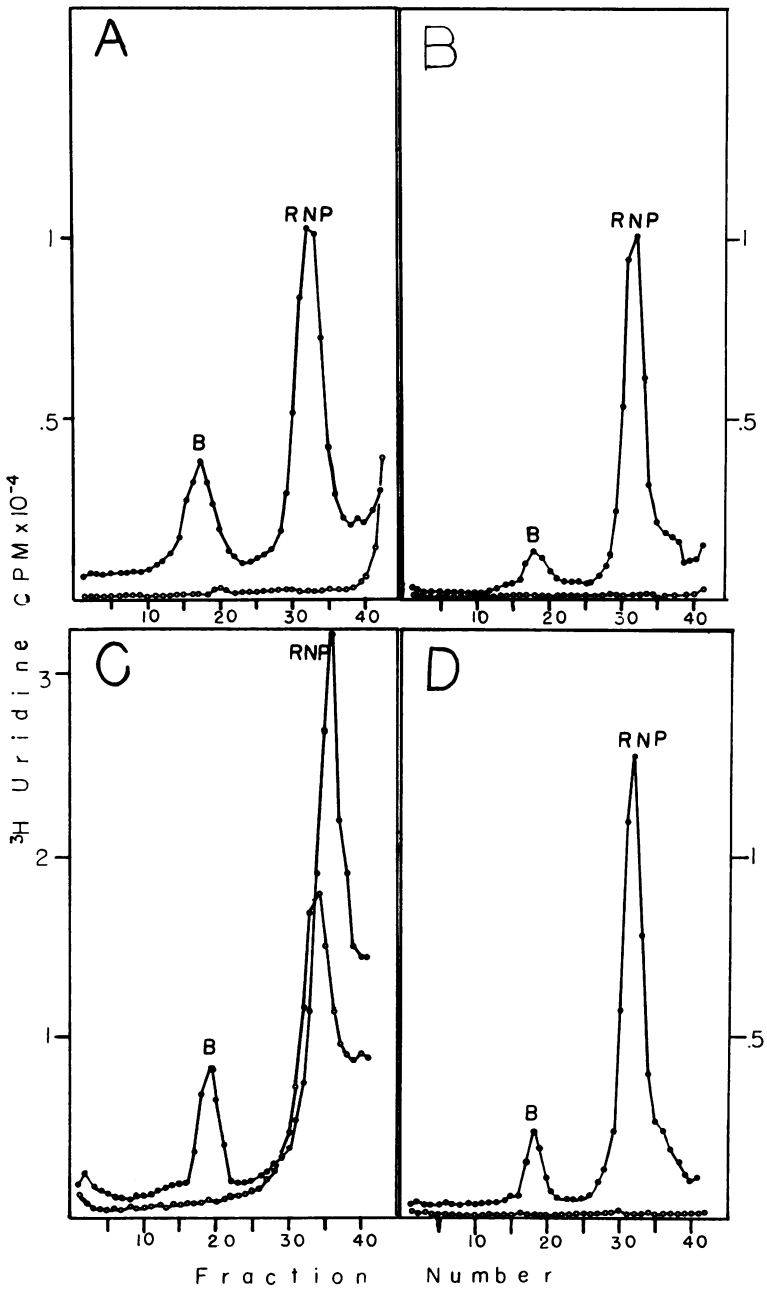

FIG. 1. *Incorporation of radioactivity by temperature-sensitive mutants of VSV into virions and ribonucleoprotein cores at permissive (31 C) and nonpermissive (39 C) temperatures. Monolayers containing 3 × 10^7 cells were infected with 50 PFU/cell and incubated for the first 5 h in medium containing 10 μg of actinomycin D per ml. At the end of 5 h, nonradioactive medium was replaced with prewarmed medium containing 10 μg of actinomycin D per ml and 20 μCi of ^{3}H-uridine per ml. Infections were permitted to continue for an additional 3 h, at which time entire samples were frozen overnight at −20 C to disrupt cells. After high speed pelleting at 100,000 × g for 2 h, samples were loaded on to a 4.8-ml 5 to 40% sucrose gradient and spun for 1 h at 30,000 rpm in an SW 50.1 rotor. Mutants representing the four Glasgow complementation groups are presented. B indicates VSV virion particles. RNP indicates ribonucleoprotein cores. Direction of sedimentation is from right to left. A, ts G11 (group I); B, ts G22 (group II); C, ts G31 (group III); and D, ts G41 (group IV). Symbols: (●) 31 C, (○) 39 C.*

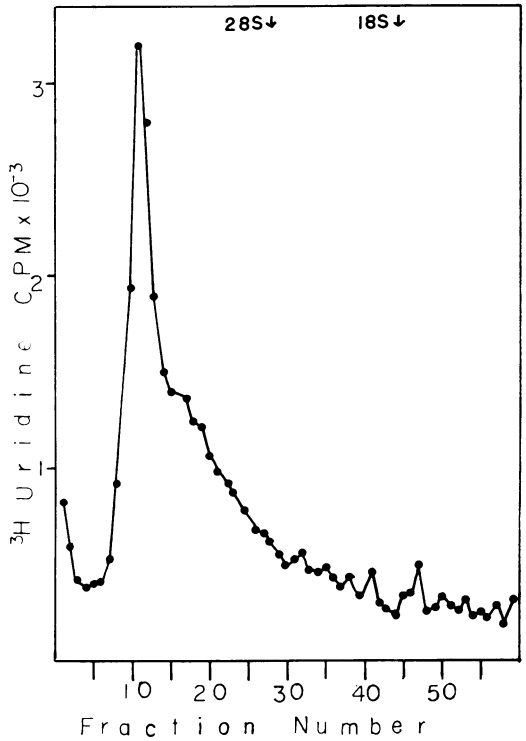

FIG. 2. *Polyacrylamide gel electrophoresis of RNA isolated from ribonucleoprotein core produced by ts G31 (group III) at the nonpermissive temperature of 39 C. The RNA sample was boiled and quick cooled before being subjected to electrophoresis for 3 h at 10 mA/gel. Position of ^{14}C BHK-21 ribosomal RNA markers indicated by arrows. Direction of electrophoresis is from left to right.*

and *ts* G41 (group IV), at 31 and 39 C, respectively. All three mutants produce viral particles and 140*S* RNP cores at 31 C; however, no particulate matter could be identified at the nonpermissive temperature of 39 C. The complete lack of formation of particulate matter in these mutants indicates that the thermally labile function is needed earlier in the viral cycle than with group III mutants.

To find differences among the three RNA$^-$ groups, we chose to try to identify any intracellular viral RNA fraction corresponding in size to species previously shown to be polysome associated during VSV infection in these mutants at the nonpermissive temperature.

Polyacrylamide gel electrophoresis of intracellular RNAs. Figure 3A shows the gel profile of RNA extracted from cells 6 h after infection with mutant *ts* G22 (group II) at the permissive temperature of 31 C. The two peaks of radioactivity which electrophoresed in the area of 18 to 13*S* correspond in size to viral transcriptase product and are presumed to be mRNA species. The same is also true for the RNA species appearing in slices 16 to 20. This long transcriptase product sediments at 28*S* on sucrose gradients (9). However, electrophoresis occurs with an apparent greater molecular weight (14).

Hybridization experiments by Robert Leamnson in our laboratory showed the above mentioned size classes of RNA to anneal to B-particle and various T-particle RNAs (manuscript in preparation). The peak of radioactivity at slice 30 is characteristically seen in our gel profiles. This peak of radioactivity is not produced in uninfected cells (Fig. 3C) and is made in the presence of cycloheximide (Fig. 3B, D). The production of this RNA in the presence of cycloheximide along with its appearance in all mutant groups including wild-type VSV strongly rules against the possibility of T-particle contamination (9). It may represent a primary transcriptional product which does not separate on sucrose gradients, and to date has been given little consideration. The peak of radioactivity at fractions 11 and 12 corresponds in size to viral RNA. We used different conditions to increase the yield of 45*S* RNA from infected cells. No method used has led to any significant increase. It, therefore, appears that viral sized RNA constitutes a relatively small proportion of the total RNA extracted from BHK-21 cells. Figure 3B shows the RNA profile of cells infected at 31 C in the presence of cycloheximide. There is reduction of RNA synthesis to ~10% of the amplified level with a loss of production of viral RNA synthesis. All RNA species corresponding in size to mRNA are clearly identifiable. Figure 3C shows the level of synthesis in uninfected cells at 39 C. To demonstrate that the level of RNA synthesis in our mutant preparation was not attributable to revertants, a monolayer of cells was infected with wild-type B particles equal in concentration to the level of revertants detectable by plaque assay at 39 C. Figure 3C also shows the level of RNA synthesis which can be attributed to revertants. Figure 3D shows the level of RNA synthesis in *ts* G22 (group II) at the nonpermissive temperature of 39 C in the presence and absence of cycloheximide. The addition of cycloheximide at 39 C has no apparent effect on the reduction of RNA synthesis. The results, therefore, indicate that *ts* G22 (group II) has a thermally stable virion-encapsidated transcriptase. The transcriptase is active at the nonpermissive temperature and produces RNA species that are similar in size to those previously reported to be polysome associated during VSV infection (8).

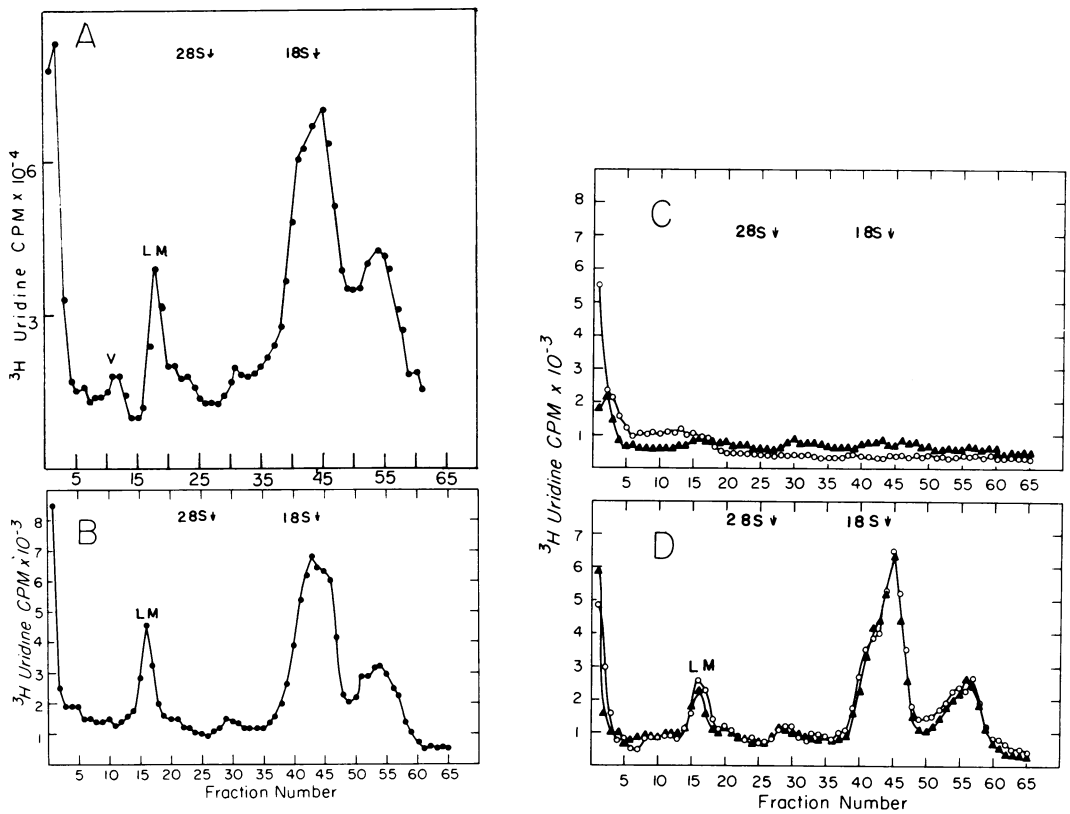

FIG. 3. *Polyacrylamide gel electrophoresis of RNA isolated from cells infected with ts G22 (Group II). Monolayers containing 3 × 10^7 cells were infected with 50 PFU/cell for a total of 6 h in the presence of 10 μg of actinomycin D per ml, 20 μCi of ^{3}H-uridine per ml, and 50 μg of cycloheximide per ml where indicated. A, RNA extracted from cells infected at the permissive temperature of 31 C; B, RNA extracted from cells infected at 31 C in the presence of cycloheximide; C, RNA extracted from uninfected cell (O), RNA extracted from cells infected with wild-type virus adjusted to the determined level of revertants in the inoculum (▲), based on plaque assay at 31 C/39 C; D, RNA extracted from cells infected at the nonpermissive temperature of 39 C in the absence (▲) and presence (O) of cycloheximide. All samples were boiled and quick cooled prior to being subjected to electrophoresis for 3 h at 10 mA/gel. Arrows indicate position of ^{14}C labeled BHK-21 ribosomal RNA markers. V indicates position of virion-sized RNA. LM indicates position of long-message RNA. Direction of electrophoresis is from left to right.*

The peaks of radioactive RNA identifiable at 31 C in infections with *ts* G41 (group IV) were identical to those synthesized by *ts* G22 (group II) and are, therefore, not shown. RNA synthesized by *ts* G41 (group IV) at 39 C is presented in Fig. 4A. All characteristic message species were synthesized at 39 C by this mutant; however, no viral RNA synthesis could be identified. No revertants in the range assayed could be detected in this preparation, and no cellular background synthesis could be demonstrated in this set of experiments (Fig. 4A). Figure 4B shows the RNA profile of *ts* G41 (group IV) at 31 and 39 C in the presence of cycloheximide. *ts* G41 (group IV), like *ts* G22 (group II), transcribes viral RNA species which are similar in size to those previously reported to be polysome associated (8); at the nonpermissive temperature, however, it fails to synthesize RNA species corresponding in size to viral RNA.

Figure 5A shows the RNA gel profiles of *ts* G11 (group I) at the permissive and nonpermissive temperature. *ts* G11 (group I) synthesizes all the characteristic RNA species normally seen in wild-type or mutant infections at the permissive temperature. The level of synthesis at 31 C in the presence of cycloheximide (not shown) is approximately the same as for *ts* G22 (group II) or *ts* G41 (group IV) under similar conditions. The gel profile of *ts* G11 (group I) at 39 C is also shown in Fig. 5A. No detectable RNA synthesis can be seen in this mutant at the nonpermissive temperature. These results more than likely indicate the existence of a thermally

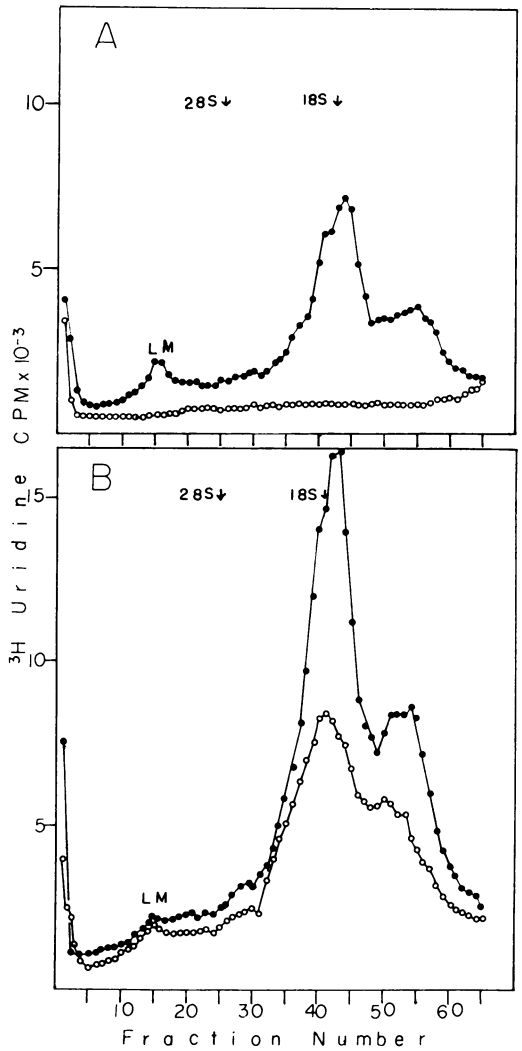

FIG. 4. *Polyacrylamide gel electrophoresis of RNA isolated from cells infected with ts G41 (group IV). Monolayers containing 3 × 10^7 cells were infected with 50 PFU/cell for a total of 6 h in the presence of 10 μg of actinomycin D per ml, 20 μCi of ^{3}H-uridine per ml and 50 μg of cycloheximide per ml where indicated. A, RNA extracted from cells infected at the nonpermissive temperature of 39 C (●), uninfected cells (○). B, RNA extracted from cells infected at the permissive temperature of 31 C (●) and the nonpermissive temperature of 39 C (○) in the presence of cycloheximide. All samples were boiled and quick cooled before being subjected to electrophoresis for 3 h at 10 mA/gel. Arrows indicate position of ^{14}C labeled BHK-21 ribosomal RNA markers. LM indicates position of long-message RNA. Direction of electrophoresis is from left to right.*

labile transcriptase apparatus in group I mutants.

The RNA gel profile of *ts* G31 (group III) at 39 C in the presence and absence of cycloheximide is presented in Fig. 5B. It can be seen that RNA synthesis in this mutant is not affected by

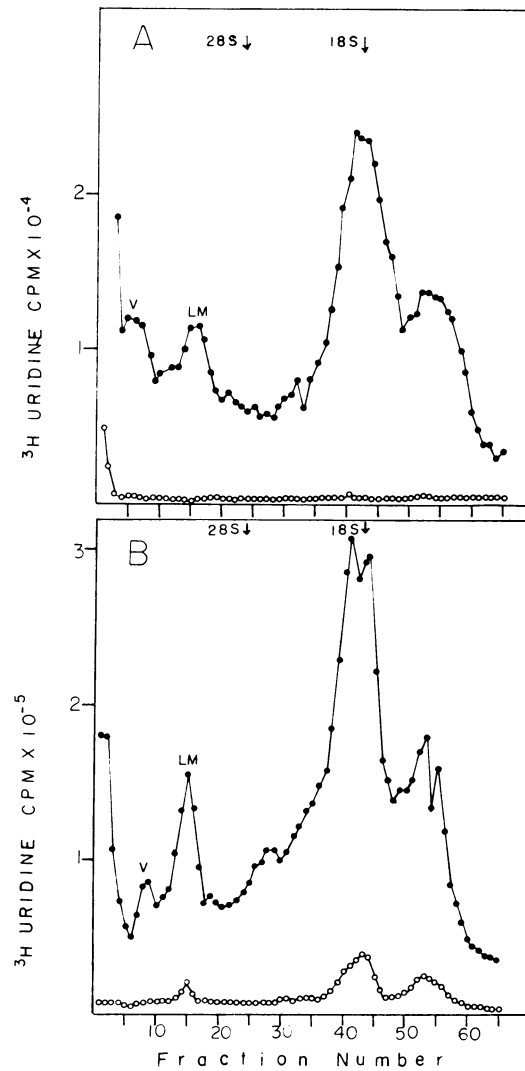

FIG. 5. *Polyacrylamide gel electrophoresis of RNA isolated from cells infected with group I and group III mutants. Monolayers containing 3 × 10^7 cells were infected with 50 PFU/cell for a total of 6 h in the presence of 10 μg of actinomycin D per ml, 20 μCi of ^{3}H-uridine per ml, and 50 μg of cycloheximide per ml, where indicated. A, RNA extracted from cells infected with ts G11 (group I) at the permissive temperature of 31 C (●), and the nonpermissive temperature of 39 C (○). B, RNA extracted from cells infected with ts G31 (group III) at the nonpermissive temperature of 39 C in the presence (○) and absence (●) of cycloheximide. All samples were boiled and quick cooled before being subjected to electrophoresis for 3 h at 10 mA/gel. Arrows indicate position of ^{14}C labeled BHK-21 ribosomal RNA markers. V indicates position of virion-sized RNA. LM indicates position of long-message RNA. Direction of electrophoresis is from left to right.*

temperature. The level of synthesis in this mutant significantly exceeds that of all other mutants tested at the permissive temperature.

DISCUSSION

The early characterization of temperature sensitive mutants derived from the Indiana serotype of VSV made no distinction between the transcriptional and replicative steps of RNA synthesis in VSV infection. The classification into RNA positive and RNA negative complementation groups of the Glasgow mutants almost certainly related to the replicative step, since the low input multiplicities and small sample size made it extremely difficult to detect RNA transcription off the incoming virion RNA. Since then, it has been shown that RNA transcription could be detected in the French mutant *ts* 0100 homologous to the Glasgow complementation group IV (18). The authors were unable to detect any significant quantities of the long mRNA species at the nonpermissive temperatures. Our profiles obtained with the *ts* G41 (group IV) show that synthesis of this species did take place. Whether this difference between *ts* G41 (group IV) and *ts* 0100 (group IV) is real or whether it is due to different methods of analysis remains to be seen.

An investigation of mutant *ts* G22 (group II) showed likewise that early transcription was not defective in group II; moreover, the RNA profile at the nonpermissive temperature was indistinguishable from the RNA profiles obtained at permissive and nonpermissive temperatures in the absence of protein synthesis (Fig. 3B, D). Despite the similarity of these profiles it is impossible to be sure that the entire group II and IV genomes have been completely transcribed at the nonpermissive temperature. Hybridization experiments with virion RNA and T-particle RNA complementary to individual mRNA species are in progress to further elucidate the nature of the transcription. Also in vivo protein synthesis is being studied to determine which mRNA species are being expressed. The RNA profiles of groups II and IV at the nonpermissive temperature did not show a species corresponding to virion RNA, indicating that the block occurred before or at the replicative step. Since virion RNA is always synthesized in appreciably smaller quantities than mRNA, we considered the possibility of losing the virion RNA during phenol extraction of total cellular RNA. To eliminate such a possibility, subparticle-bound RNA was isolated from the total cytoplasmic content and enriched by ultracentrifugation of extracts from large volumes of cells. The RNA profiles of these preparations for each complementation group at both temperatures were also investigated by this procedure. Groups II and IV did not yield any virion-like RNA at the nonpermissive temperature, while relatively large amounts were recovered at the permissive temperature.

A quantitative comparison between the RNA profiles obtained at the permissive temperature in the presence of cycloheximide and at the nonpermissive temperature with and without cycloheximide indicated that amplification of transcription is blocked in groups II and IV. The most obvious reason for the lack of amplification is the lack of synthesis of virion stranded RNA, limiting the transcription to the incoming template. Other explanations, e.g., a block in pathways leading to synthesis of additional transcriptase or other proteins required for transcription, are less likely, although possible.

The analysis of RNA synthesis at the nonpermissive temperature in group III confirmed the original findings of the Glasgow group (3). As far as the resolution permits, all components of mRNA as well as the virion RNA were synthesized. Among the four Glasgow complementation groups the only truly RNA negative group seems to be group I. This is clearly demonstrated by the data in Fig. 5A. At the nonpermissive temperature no virus induced RNA synthesis was found either in the total cellular extracts or in the particle-bound extracts. These results are also in agreement with the data obtained with the corresponding French mutant *ts* 05 (group I) (18).

The original report of three RNA negative complementation groups implied that at least three functions were required in transcription and that a physical exchange of functional molecules between incoming virions was necessary for complementation. In view of our present finding of but a single transcriptional RNA negative group such assumptions are no longer necessary, and the question remains whether complementation between a RNA negative group and any of the transcription positive groups could occur in this manner, or whether newly synthesized transcriptase utilizes the RNA of group I virions as a template in the process of complementation. It should be noted that if there is no efficient mechanical exchange of the functional moieties of the transcriptional apparatus prior to protein synthesis, no complementation could occur between mutants with lesions in these different subunits. In that case, group I could be a complex group of mutants. The existence of a relatively large number of mutants in this group, as reported (16), would also be understandable in the light of this interpretation. We are in the process of

looking into this possibility through an investigation of the behavior of other members of the group.

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