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RNA Synthesis by Vesicular Stomatitis Virus and a Small Plaque Mutant: Effects of Cycloheximide

GAIL W. WERTZ AND MYRON LEVINE

Department of Human Genetics, University of Michigan Medical School, Ann Arbor, Michigan 48104

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The synthesis of viral RNA by wild-type vesicular stomatitis virus (L_1VSV) and a small, plaque-size mutant (S_2VSV) was studied in vitro and in chicken embryo (CE) and mouse L-cell cultures. Virus-specific RNA synthesized in CE or L cells infected with either L_1 or S_2VSV at low multiplicity was of the same size classes, 12 to 15*S*, 28*S*, and 38*S*. The major differences were in the proportion of RNA produced of each size class. L_1VSV always synthesized larger proportions of 38*S* RNA, and S_2VSV produced larger proportions of 12 to 15*S* RNA. Both S_2 and L_1VSV exhibited RNA transcriptase activity in vitro and in cell culture. The products of the in vitro reaction were the same, 12 to 15*S* for both. The products of the virion-associated transcriptase in CE or L-cell cultures in the presence of cycloheximide were also the same for both viruses but differed from the in vitro products in that 28*S* and 12 to 15*S* RNA were made. The effects of addition of cycloheximide at various times after infection demonstrated that new protein synthesis is required early (0-2 h) for both S_2 and L_1VSV to initiate and maintain the normal rate of viral RNA synthesis. However, the overall rate of RNA synthesis in L_1VSV infections became independent of protein synthesis after 2 h whereas the rate in S_2VSV infections did not. With either virus, synthesis of 38*S* RNA did not occur in the absence of protein synthesis. Moreover, continuous 38*S* RNA production required continuous protein synthesis. Production of 38*S* RNA ceased within 30 min after addition of cycloheximide to S_2- or L_1VSV-infected CE or L cells that had already begun to synthesize the 38*S* form. The cycloheximide-induced cessation of 38*S* RNA synthesis was accompanied by a marked increase in production of 12 to 15*S* and 28*S* RNA in L_1VSV-infected cells, but no increase in synthesis of small RNA species occurred in S_2VSV-infected cells.

Previously, we reported the isolation of a large plaque strain of the Indiana serotype of vesicular stomatitis virus, called L_1VSV (wild type) and a small plaque-size mutant, S_2VSV (23). Both viruses elicited equivalent titers of circulating interferon in mice at 12 h, although the wild-type virus was approximately 1,000 times more lethal than the small plaque mutant (23). In both chicken embryo (CE) and mouse L-cell cultures, the L_1 and S_2 mutants differed in ability to shut off host RNA and protein synthesis, in ability to stimulate synthesis of interferon, and in efficiency of virus production (20, 21). L_1 produced an average yield of 150 PFU/cell in L cells, whereas S_2 yielded an average of 6 PFU/cell. Both viruses replicated more efficiently in CE cells, giving an average yield of 850 PFU/cell for L_1 and 130 PFU/cell for S_2. The relative yield of the two viruses was not related to the effects of interferon produced during the infection.

The kinetics of RNA synthesis during L_1 and S_2 infections of L cells also were found to differ (20). The rate of RNA synthesis directed by S_2 in L cells reached a maximum at 2 to 3 h after infection and declined gradually over the next 4 h. Very little viral RNA was synthesized by 8 h after infection. In contrast, RNA synthesis directed by L_1 did not reach a maximal rate until 6 to 8 h postinfection, although there was an early peak at 2 h. In CE cells, there was a steady increase in viral RNA synthesis with both viral strains, until a maximum rate was reached at approximately 3 to 5 h after infection. Progeny

virus particles first appeared at about 2 h in both infections.

In view of these observations, a detailed examination of L_1 and S_2 viral RNA synthesis was undertaken in an attempt to explain the differences in virus yields. Since VSV has an RNA-dependent RNA transcriptase that functions in vitro (1, 2, 3) and in cell culture in the presence of cycloheximide (9, 12), it was possible to examine transcription apart from other viral replicative events.

This paper reports the examination of RNA synthesis directed by S_2 and L_1VSV both in vitro and in CE and L-cell cultures. Evidence is presented indicating that the reduced viral yield of S_2VSV is not due to a defect in the virion-associated RNA transcriptase but is the result of some defect in subsequent viral RNA synthetic events. In addition, we show that continuous protein synthesis is necessary for production of 38*S* viral RNA.

MATERIALS AND METHODS

Media and cell cultures. Eagle minimal essential medium supplemented with 10% heat-inactivated calf serum was used for growth and maintenance of all cell cultures.

Primary cultures of CE cells were prepared from 11-day-old embryos. The embryos were minced and treated with trypsin, and after centrifugation 1 ml of packed cells was suspended in 650 ml of growth medium. A sample (4 ml) of this material was used to seed 60-mm petri dishes. These cultures were used in experiments after 48 h of incubation at 37 C in a humidified atmosphere of 5% CO_2. At this time each plate contained approximately 2×10^6 cells.

A continuous line of mouse L cells (clone 929) was maintained as monolayers and was subcultured every 7 days with one change of medium. Cultures in petri dishes were prepared by scraping the cells from the glass with a rubber policeman, suspending them in growth medium, and seeding 2×10^6 cells per 60-mm petri plate. These cultures were used after incubation for 12 h in a humidified atmosphere of 5% CO_2 at 37 C.

A continuous line of baby hamster kidney (BHK) cells was grown in monolayer cultures and used for propagation of virus.

Virus. The Indiana strain of VSV was propagated in CE or BHK cell monolayers. Cell cultures were inoculated with virus at a low multiplicity of infection (0.001) to avoid formation of incomplete, autointerfering T particles (6, 8). After an adsorption period of 60 min at 37 C, media were added and the cultures were incubated for 18 h. Infected culture fluids were harvested, pooled, and centrifuged at $1{,}000 \times g$ for 15 min to remove cellular debris. The clarified fluids were centrifuged at $54{,}450 \times g$ for 90 min at 5 C. Virus pellets were resuspended in EDTA to 1/100 the volume of the starting material and treated for 30 s in a 250-W, 10-kcycle Raytheon sonic oscillator to disaggregate clumps of virus. Concentrated virus suspensions were further purified by treatment with fluorocarbon by the method of Hackett et al. (6). The partially purified virus concentrates were layered on preformed, linear 15 to 45% sucrose gradients and centrifuged at 20,000 rpm for 105 min at 10 C in a Spinco model L-2 rotor (SW25.1). Fractions were collected from the bottom of the gradient with monitoring at 280 nm in a continuous flow cell of a Gilford recording spectrophotometer. A single peak of absorbance at 280 nm and a corresponding single visible band indicated the presence of only infectious B particles.

Chemicals. Cycloheximide, purchased from Nutritional Biochemicals Corp., was diluted in medium and used at a final concentration of 50 μg/ml. Actinomycin D was purchased from Schwarz/Mann and used at a final concentration of 2 μg/ml. Uridine-2-^{14}C (specific activity, 58 mCi/mmol), uridine-5-6-^{3}H (specific activity, 43 Ci/mmol), and ^{3}H-UTP (specific activity, 15 Ci/mmol) were from Schwarz/Mann.

Incorporation of labeled precursors. Viral RNA synthesis was studied in monolayer cultures containing 2×10^6 CE or L cells per dish. Cells were infected at the desired multiplicity of virus in the presence of 2 μg of actinomycin D per ml. This concentration of actinomycin inhibits 98% of host cell RNA synthesis within 30 min (20). Uninfected control cultures were prepared simultaneously in all experiments. After a 30-min absorption period at 37 C, the fluids containing the inoculum were drawn off and replaced with 2 ml of fresh medium containing actinomycin D. A sample (2 ml) of culture medium containing radioactive uridine (2-5 μCi/ml) was added to each cell monolayer at indicated intervals. Total cellular incorporation of label was measured as follows. After the labeling period, the fluid containing the label was discarded and the cells were washed twice with phosphate-buffered saline (PBS), resuspended by scraping in PBS, and mixed with an equal volume of cold 10% trichloracetic acid. The trichloroacetic acid-precipitable fraction was collected by suction filtration on Whatman GF/c filters. The dry filters were treated with 0.5 ml of NCS solubilizer (Amersham/Searle Corp.) for 30 min at 22 C. A sample (10 ml) of toluene-based scintillation mixture was added to each solubilized sample. The samples were counted in a Packard liquid scintillation spectrometer.

Sucrose gradient analysis of cytoplasmic RNA. Cells exposed to radioactive uridine as described above were washed and suspended in reticulocyte standard buffer (RSB: 0.01 M Tris [pH 7.4]-0.01 M NaCl-0.0015 M $MgCl_2$). The cells were disrupted by 15 to 20 strokes of a motorized Duall homogenizer. Efficiency of disruption was >95% and was always monitored by phase-contrast microscopy. Intact nuclei were removed by centrifugation at $750 \times g$ for 2 min. The supernatant fluid (cytoplasmic extract) was made 1% with respect to sodium dodecyl sulfate (SDS). These solubilized extracts were analyzed by velocity sedimentation in 15 to 30% (wt/wt) linear sucrose gradients containing 0.01% SDS in buffer (0.01 M Tris, pH 7.5; 0.1 M NaCl; 0.001 M EDTA). Centrifugation was carried out at 20 C for 15.5 h at 23,000 rpm in the SW25.1 rotor in a Spinco model L-2.

After centrifugation, the gradients were frac-

tionated and monitored for absorbance at 260 nm by pumping from the bottom of the tube through the continuous flow cell of a Gilford recording spectrophotometer. Radioactivity present in the acid-insoluble portion of each fraction was assayed as described above. Ribosomal 18*S* and 28*S* RNA present in the cytoplasmic extracts and detected by absorbance at 260 nm were used as markers in all gradients.

In vitro RNA polymerase assay. Virion-associated RNA polymerase activity was assayed by incubating purified virus particles in a standard reaction mixture consisting of: 15 μmol of Tris-hydrochloride buffer, pH 7.5; 1.5 μmol each of the unlabeled ribonucleoside triphosphates, ATP, GTP, and CTP; 0.02 μmol of ^{3}H-UTP (100 μCi/μmol); and 0.25 mg of Triton N-101 in a total volume of 300 μliters. Reactions were allowed to proceed in sealed test tubes and were terminated by addition of 0.5 ml of 0.08 M sodium pyrophosphate. Carrier yeast RNA and cold 10% trichloroacetic acid were added, and acid-precipitable material was collected on GF/c filters and assayed for radioactivity as described above.

RESULTS

Sedimentation properties of RNA from purified S_2 and L_1 virions. No differences in rate of sedimentation in sucrose or in particle morphology, as determined by electron microscopy, have been detected between L_1 and S_2 virions (Youngner and Wertz, unpublished data). In addition, the sedimentation coefficients of RNA from infectious S_2 or L_1 virions were examined and found to be similar. S_2 and L_1 were propagated in BHK cells infected at low multiplicity (0.001) in the presence of actinomycin D and ^{3}H-uridine. The respective virus pools were harvested and purified by banding in sucrose gradients. The single peak of virus present was collected and dialyzed against RSB to remove sucrose. Samples of purified S_2 and L_1 virus were made 1% with respect to SDS and the released labeled RNA was analyzed by velocity sedimentation in sucrose. A single peak of RNA which sedimented at 38*S* was present in both S_2 and L_1 preparations. This finding indicated no difference in the size of the RNA released from infectious S_2 and L_1 virions. This observed sedimentation coefficient is in agreement with previous estimates (36–43*S*) for RNA molecules released from VSV B particles (4, 10, 14, 16, 18).

Comparison of L_1- and S_2-virus-specific RNA synthesis in cell culture. Virus-specific RNA was examined in cells infected with L_1 or S_2 to detect any variations in size or amount of RNA synthesized. Actinomycin D-treated CE or L cells were infected with an input multiplicity of three. At various times after infection cells were exposed to ^{3}H-uridine. At the end of the labeling period the cells were harvested and cytoplasmic extracts were prepared and solubilized by addition of SDS. RNA synthesized during intervals from 0 to 2, 2 to 4, and 4 to 6 h postinfection was examined on sucrose gradients. In all cases, uninfected, control cultures were prepared simultaneously. The radioactivity present in the gradients from control cultures was background level except for the 4*S* area, indicating that the actinomycin D treatment used was effective in inhibiting cellular RNA synthesis. The label present in the 4*S* area of the control gradients was apparently due to terminal labeling of transfer RNA which is

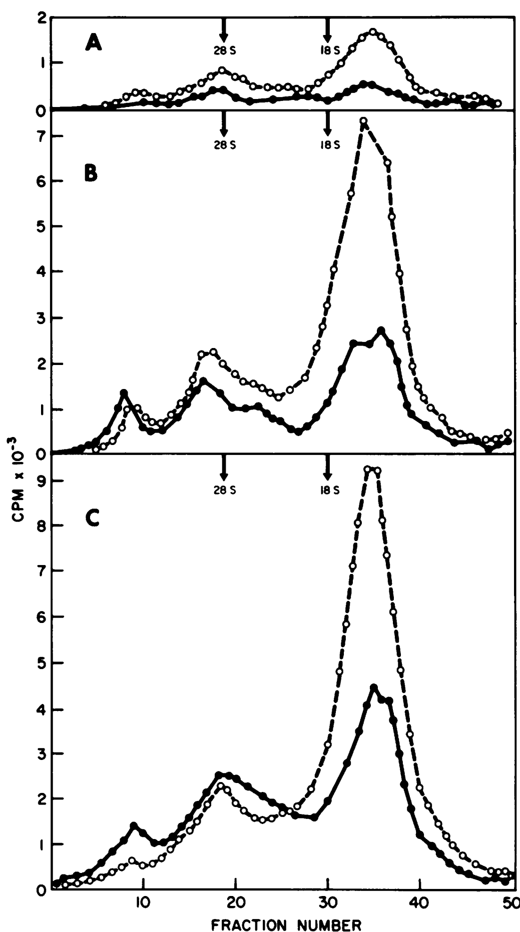

FIG. 1. *Velocity sedimentation analysis of RNA synthesized in actinomycin D-treated CE cells after infection with S_2VSV (○) or L_1VSV (●). Cells were exposed to 4 μCi of ^{3}H-uridine for designated intervals after infection with S_2 or L_1 at a multiplicity of 3. After the labeling period, cytoplasmic extracts were prepared, made 1% with SDS, and analyzed by centrifugation in sucrose-SDS gradients for radioactivity incorporated into acid-insoluble material 0 to 2 h postinfection (a), 2 to 4 h postinfection (b), and 4 to 6 h postinfection (c).*

resistant to the presence of actinomycin D (5). Control counts have been subtracted from experimental values in all figures. Figure 1 shows the profile of virus-specific RNA species extracted from S_2- or L_1-infected CE cells.

The RNA species synthesized in cells infected with L_1 at low multiplicity during each time interval fall into three major size classes: (i) small, 12 to 15*S*; (ii) intermediate, 28*S*; and (iii) large, 38*S*. The total amount of RNA synthesized during each of the intervals increased from early to late times. Table 1 shows the relative amounts of RNA in the three major size classes synthesized during the observed time intervals. These values were obtained by summing the areas under the peaks of the profiles in Fig. 1, but are representative of at least three other separate experiments. In L_1-infected cells the 12 to 15*S* species constituted the largest fraction of total RNA synthesized during all time intervals, and the proportion represented by 12 to 15*S* material was approximately constant with time. Intermediate 28*S* RNA was synthesized at a fairly constant level at all times. Previous work (7, 13, 22) suggests that 12 to 15*S* and 28*S* RNA can function as mRNA. The 38*S* RNA, which we presume corresponds to mature virion RNA, was synthesized in constant or slightly increasing amounts from early to late times. These results for wild-type, L_1 RNA synthesis are in basic agreement with those reported by others (7, 13, 15, 16, 17, 18, 19, 22) with variations probably due to cell type, strain of virus, multiplicity of infection, and experimental procedure.

RNA synthesis in S_2-infected cells was characterized by synthesis of the same size classes of RNA seen in L_1 infections. Differences were evident, however, in the amount and time of synthesis of these RNA classes. As with L_1, the total amount of RNA synthesized in S_2-infected cells during each of the observed intervals increased from early to late times (Fig. 1). However, the accumulation of radioactive uridine into total viral RNA was greater in CE cells infected with S_2 than in those infected with L_1. In contrast to L_1 infections, the proportions of 12 to 15*S* RNA steadily increased while the proportions of 28*S* and 38*S* RNA decreased. The largest proportion of S_2 38*S* RNA was synthesized early and then decreased to less than 3% of the total during the 4 to 6-h interval. The proportions of each of the three main size classes of RNA synthesized during each interval by L_1 and S_2 were the same in CE and L cells. In summary, the major difference between L_1 and S_2 infections was in the proportions of RNA produced of each size class. L_1 synthesized larger proportions of 38*S* RNA and, in contrast, S_2 produced greater proportions of 12 to 15*S* material. The reduced amounts of intracellular 38*S* RNA in S_2 infections compared with L_1 infections was not due to more rapid maturation and release of S_2 virions as revealed by examination of extracellular fluids for released, labeled 38*S* RNA.

In vitro RNA synthesis. To determine whether the altered pattern of S_2 RNA synthesis was due to an altered or defective transcriptase (1, 2, 3), S_2 and L_1 virions were examined for in vitro RNA polymerase activity. Equal amounts of virion protein containing equivalent titers of plaque-forming units were incubated in the standard reaction mixture described above. Both S_2 and L_1 particles have an enzymatic activity that stimulates incorporation of labeled ribonucleotides into RNA. The incorporation was linear with time and with increasing amounts of viral protein for both viruses and resulted in approximately equal amounts of acid-insoluble product.

A difference in optimal conditions for expres-

TABLE 1. *Distribution of labeled RNA among three major size classes*

Hours post-infection	Mutant	Small (12-15*S*)		Intermediate (28*S*)		Large (38*S*)		Total ^{3}H-uridine incorporated during each interval (counts/min)
		Counts/min	%	Counts/min	%	Counts/min	%	
0-2	L_1	4,208	58	2,292	31	794	11	7,294
	S_2	14,219	62	6,640	29	1,827	8	22,686
2-4	L_1	23,066	55	13,383	32	5,224	13	41,673
	S_2	56,541	70	19,906	24	4,896	6	81,339
4-6	L_1	43,276	57	25,631	34	6,621	9	75,528
	S_2	73,888	76	20,249	21	2,791	3	96,925

sion of in vitro enzymatic activity was observed. Equal amounts of virus (15 μg of protein) were incubated in the standard reaction mixture for 60 min at the temperatures indicated in Fig. 2 and then assayed for total radioactivity incorporated into acid-precipitable material. The L_1 polymerase activity displayed a temperature optimum of 32 C, whereas S_2 polymerase exhibited optimal activity at 28 C. In addition, a series of samples was assayed in which the pH of the reaction mixtures was varied but the temperature was held constant (Fig. 3). Both viruses displayed a rather broad optimum of activity, with a peak at pH 7.6 for L_1 and peaks at 7.2 and 7.6 for S_2. These differences are not surprising, perhaps, in a preparation of whole virus particles. For the present, we were satisfied to learn that both L_1 and S_2 virions did have transcriptase activity.

The products of the in vitro reactions were examined on sucrose-SDS gradients. The standard reaction mixture was incubated for 60 min with 15 μg of either L_1 or S_2. The reaction was terminated by addition of SDS to a final concentration of 1% and the solubilized mixtures were immediately layered on 15 to 30% sucrose-SDS gradients and processed as described in Materials and Methods. Controls containing no virus were processed simultaneously. The only RNA synthesized by S_2 or L_1 under these conditions was approximately 12 to 15*S*. These results concerning the size of the in vitro product are in agreement with those of previous workers (2, 3), who also demonstrated that the products were complementary to virion RNA and that therefore the enzyme could properly be termed a transcriptase.

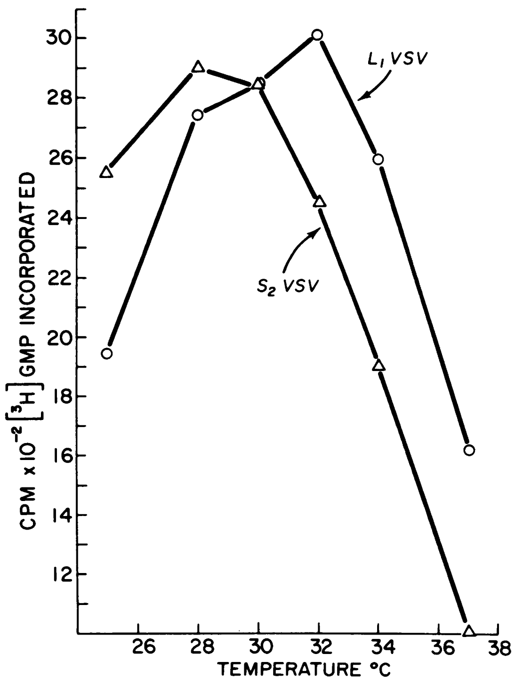

FIG. 2. *S_2VSV and L_1VSV RNA polymerase activity as a function of temperature. Equal quantities of L_1 or S_2 (15 μg of protein) were incubated for 60 min in the standard reaction mixture at each of the indicated temperatures. At the end of the incubation period, reactions were terminated and assayed for radioactivity incorporated in acid-insoluble product.*

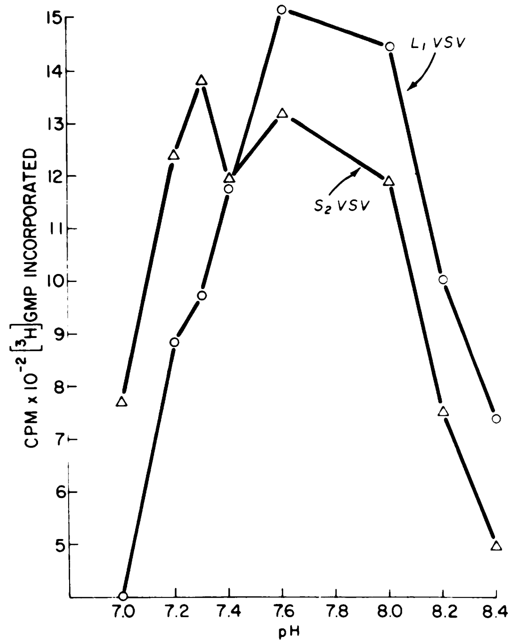

FIG. 3. *S_2VSV and L_1VSV RNA polymerase activity as a function of pH. Equal quantities of S_2 or L_1 (15 μg of protein) were incubated for 60 min at 30 C in the standard reaction mixture containing 15 μmol of Tris buffer at the different pH values indicated. Reactions were terminated and assayed for radioactivity incorporated into acid-insoluble product.*

Virion-directed RNA synthesis in absence of protein synthesis. Having determined that L_1 and S_2 particles both possess an RNA transcriptase activity that functions in vitro, the following experiments were designed to examine the activity of the L_1 and S_2 virion-associated transcriptase in CE and L cells. Actinomycin D-treated cells were infected with equal multiplicities of L_1 and S_2 in the presence or absence of cycloheximide (50 μg/ml) and then exposed to radioactive uridine. This concentration of cycloheximide inhibits 96% of CE or L-cell protein synthesis within 20 min. At intervals after

infection, cells were harvested and assayed for incorporation of radioactive uridine into acid-insoluble product. It is presumed that the only RNA synthesis in the presence of cycloheximide would be due to the virion-associated transcriptase (9, 12). Figure 4a shows that cumulative RNA synthesis in L cells infected with L_1 or S_2 in the presence of cycloheximide was approximately 8% of that in the absence of the drug. Here, as in all subsequent experiments, uninfected control cultures were prepared simultaneously, and control values have been subtracted from experimental values in all figures. As observed previously (Fig. 1), in CE cells infected with equal multiplicities of S_2 or L_1, the total amount of RNA synthesized was greater in S_2-infected cells than in L_1-infected cells. This is true also for the amount of RNA synthesized in the presence of cycloheximide; L_1 and S_2 RNA production was 5 and 10%, respectively, of the total amount of viral RNA synthesized in the absence of the drug (Fig. 4b).

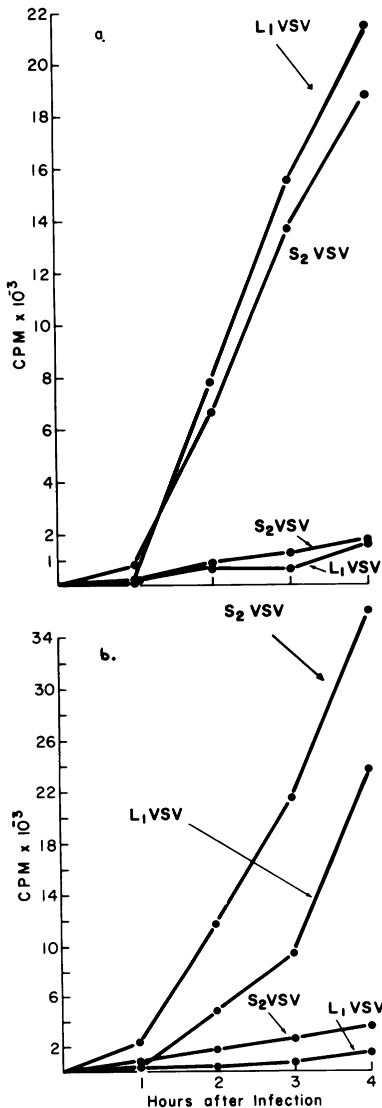

FIG. 4. *Cumulative RNA synthesis in L (a) or CE (b) cells in the absence (upper curves) or presence (lower curves) of cycloheximide added at the time of infection. Actinomycin D-treated cells were infected with an equal multiplicity (17) of L_1 or S_2 and exposed to ^{14}C-uridine. At indicated intervals cells were harvested and assayed for radioactivity incorporated into acid-insoluble material.*

The RNA produced in the absence of protein synthesis was analyzed by velocity sedimentation in sucrose-SDS gradients. The purpose of this analysis was to compare the species of RNA made by S_2 and L_1 under these conditions and to see whether these species of RNA corresponded in any way to viral RNA transcribed in vitro or to viral RNA synthesized in cells in the presence of protein synthesis.

Figure 5 shows that predominately 12 to 15*S* and 28*S* RNA molecules were synthesized during the first 3.5 h after L_1 and S_2 infection of CE or L cells in the presence of actinomycin D and cycloheximide. No 38*S* RNA was formed. These results confirm the previous observation of Marcus et al. (12), that VSV RNA synthesis does occur in cells in the absence of protein synthesis, and also the report of Huang and Manders (9), that intracellular RNA transcription results in production of both 28*S* and 12 to 15*S* species of RNA, whereas in vitro synthesis results only in the production of RNA smaller than 15*S*. Additionally, these reports (9, 12) demonstrated that the RNA produced in the presence of cycloheximide was complementary to virion RNA.

Viral RNA synthesis in the presence of cycloheximide added at intervals after infection. The following experiments were performed to determine the effect of inhibition of protein synthesis on the rate of viral RNA synthesis that is in progress. Actinomycin D-treated L and CE cells were infected with L_1 or S_2RNA at a multiplicity of 10. At intervals starting at the end of the adsorption period, duplicate samples of each of the infections were labeled with ^3H-uridine for 20 min. The control rates of viral RNA synthesis in the four infections are shown in Fig. 6 (solid lines). At the times indicated by arrows, cycloheximide (50 μg/ml) was added to a series of duplicate cultures which were subsequently examined at intervals for the rate of uptake of labeled uridine. The results presented in Fig. 6 (broken

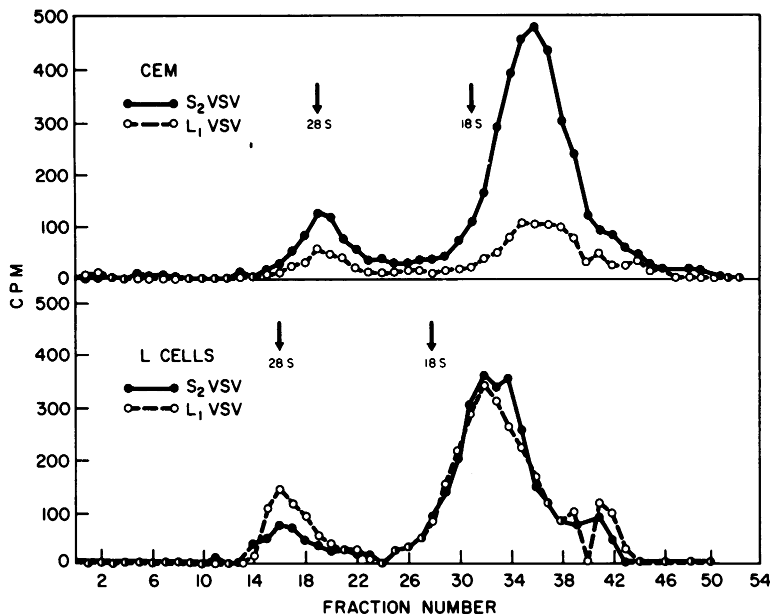

FIG. 5. *Velocity sedimentation analyses of RNA made in S_2VSV- or L_1VSV-infected CE or L cells in the presence of cycloheximide. Actinomycin D-treated cells were infected in the presence of cycloheximide with an equal multiplicity (17) of L_1 or S_2. ^{14}C-uridine was added and the cultures were incubated at 37 C for 3.5 h. Cytoplasmic extracts were made 1% with SDS and analyzed on sucrose-SDS gradients for radioactivity incorporated into acid-insoluble material.*

lines) show the effect of cycloheximide on the rate of L_1 or S_2 synthesis. In either cell type infected with S_2, cyclohemixide prevented any further increase in the rate of RNA synthesis. With continued incubation with cycloheximide the rate either remained constant or declined. Similarly, addition of cycloheximide during the first 1.5 to 2 h after infection of CE or L cells with L_1 prevented the subsequent increase in the rate of RNA synthesis that was observed in untreated control cultures. Surprisingly, however, addition of cycloheximide to L_1-infected cells after 1.5 to 2 h postinfection had no inhibitory effect on the subsequent rate of RNA synthesis. Rather, the rate of L_1 RNA synthesis increased compared with untreated controls when the drug was added after 1.5 to 2 h. Multiplicity of infection had no effect on this phenomenon. S_2 or L_1 infections of CE or L cells carried out at multiplicities ranging from 1 to 100 gave essentially similar results.

In view of these results it was of interest to determine the species of RNA synthesized under these conditions. Because we have previously demonstrated that no 38*S* RNA is made when protein synthesis is inhibited from the start of infection, the following experiments were designed to examine RNA synthesis in the presence of cycloheximide added at a time after synthesis of 38*S* RNA had already begun.

Actinomycin D-treated CE cells were infected with L_1 or S_2 at a multiplicity of 3. At 2 h postinfection, one set of cultures was exposed to ^{3}H-uridine for 1 h. This group of cells was then harvested, and cytoplasmic extracts were analyzed on sucrose gradients for species of labeled RNA. At 3 and 4 h postinfection, cycloheximide was added to one set of cultures while another set remained untreated. Both sets of cultures were exposed to labeled uridine 30 min after the addition of cycloheximide. One hour after addition of ^{3}H-uridine (4.5 and 5.5 h postinfection), the cultures were harvested and cytoplasmic extracts were examined in sucrose gradients.

The profile of RNA species synthesized in infected cells labeled 2 to 3 h after infection was identical to the results shown in Fig. 1B. Both L_1 and S_2 were synthesizing 38*S* RNA at the time cycloheximide was added. Figure 7 compares RNA synthesized in L_1-infected CE cells in the presence or absence of cycloheximide. Similar results were obtained in L cells. In the absence of cycloheximide, 12 to 15*S*, 28*S*, and 38*S* RNA species were synthesized. Total inhibition of 38*S* RNA synthesis occurred within 30 min after addition of cycloheximide. The cessation of 38*S* RNA synthesis was accompanied by increased production of 12 to 15*S* and 28*S* RNA. Synthesis of 12 to 15*S* material in cyclohexi-

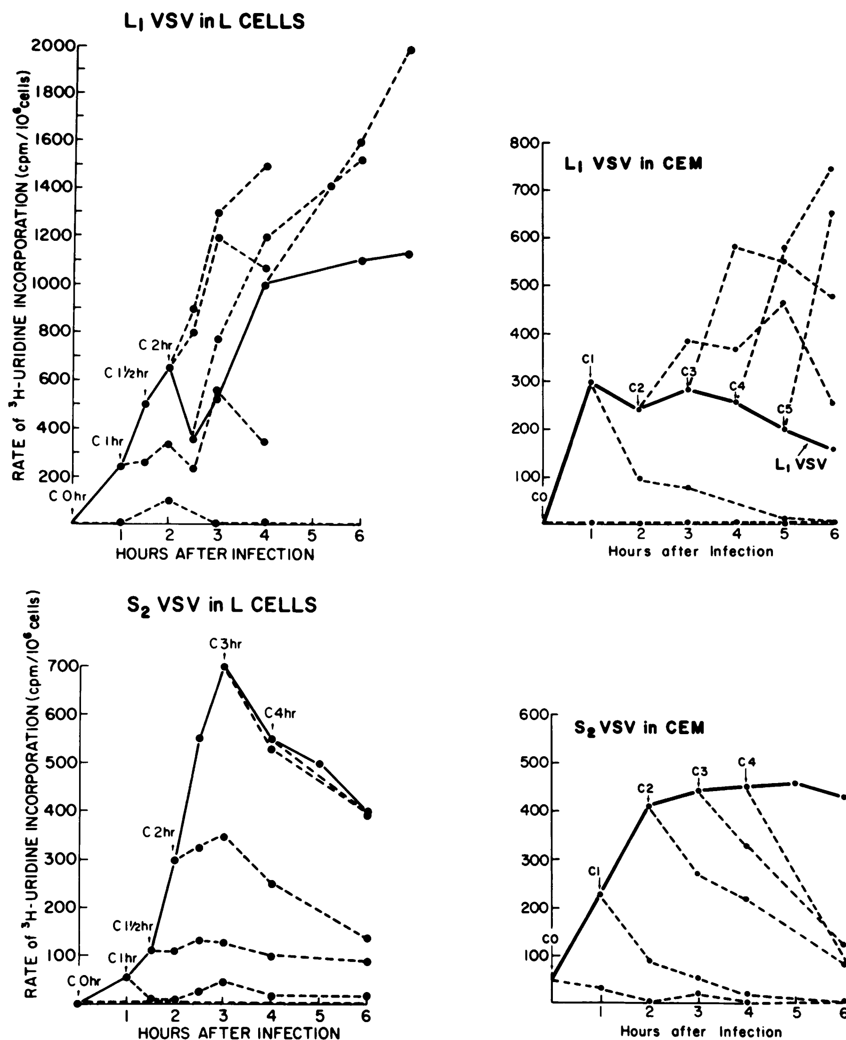

FIG. 6. *Effect of cycloheximide on the rate of S_2VSV or L_1VSV RNA synthesis. CE- or L-cell monolayers were infected with S_2 or L_1 at a multiplicity of 10. At intervals after infection cells were exposed for 20 min to ^{3}H-uridine and then assayed for incorporation of radioactivity into acid-insoluble material. Starting from time zero, at times indicated by arrows, cycloheximide (50 μg/ml) was added to duplicate sets of cultures. At the indicated intervals after addition of cycloheximide these cultures were exposed for 20 min to ^{3}H-uridine in the presence of the drug and subsequently assayed for radioactivity present in acid-insoluble material. The rate of viral RNA synthesis in the absence (●——●) or presence (● - - ●) of cycloheximide is shown.*

mide-treated cells was increased by 110 to 130% of that in the absence of the drug. Synthesis of 28*S* material increased by approximately 20% of that in untreated cells.

Similarly, addition of cycloheximide to S_2-infected CE cells (Fig. 8) resulted in shutoff of 38*S* RNA synthesis. However, in contrast to the results observed in L_1 infections no increase in synthesis of 12 to 15*S* or 28*S* RNA species occurred. In fact, a decrease of 5 to 20% was observed for both classes of RNA. Here again, similar results were obtained in L cells.

It should be noted that in both the S_2 and L_1 infections a change in the shape of the 28*S* peak occurred. In the absence of cycloheximide this peak was broad, indicating heterogeneity. RNA synthesized after addition of cycloheximide, however, sedimented in this area in a sharp, homogeneous peak.

DISCUSSION

The results presented here suggest that the reduced particle yield of S_2VSV compared to L_1VSV is not traceable to a defect in the

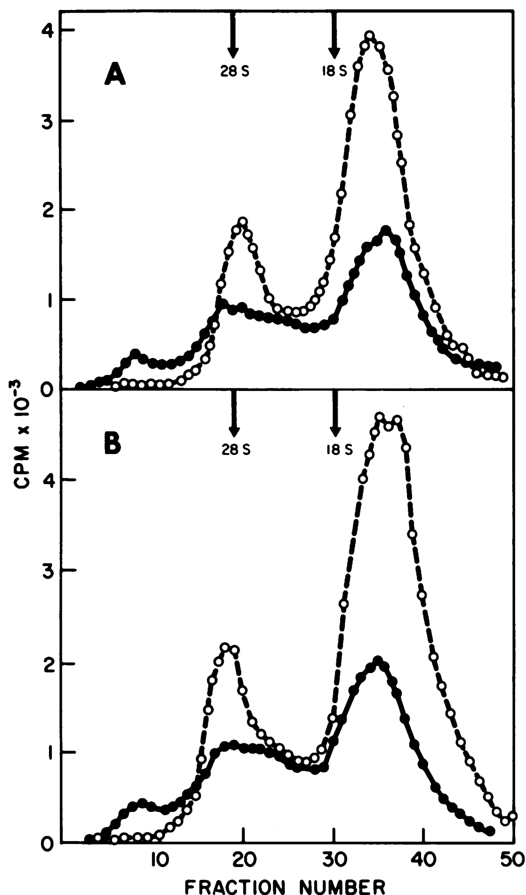

FIG. 7. *Velocity sedimentation analysis of L_1VSV RNA synthesized in CE cells in the absence (●) or presence (○) of cycloheximide (50 µg/ml). Duplicate sets of actinomycin D-treated CE cells were infected with L_1 at a multiplicity of 2. At 3 (a) or 4 (b) h postinfection cycloheximide was added to one set of cultures. Thirty minutes after addition of cycloheximide both sets of cultures were exposed for 60 min to 4 µCi of ^{3}H-uridine. At the end of the labeling period cytoplasmic extracts were prepared, made 1% with SDS, and analyzed by centrifugation in sucrose-SDS gradients for radioactivity in acid-insoluble material.*

virion-associated RNA transcriptase. Both S_2 and L_1 exhibit transcriptase activity in vitro and in cell culture. Further, the products of the in vitro reaction have similar sedimentation coefficients (12 to 15*S*) for both S_2 and L_1. The RNA products synthesized by the transcriptase in CE or L-cell cultures in the presence of cycloheximide are also the same for both S_2 and L_1 but differ from the in vitro products in that 12 to 15*S* and 28*S* RNA are made. Therefore, by the parameters we have measured, S_2 virion-associated transcriptase functions at least as well as that of L_1 and, apparently, has no defect that would account for the reduced yield of S_2 virions.

When total viral-specific RNA synthesis was examined in CE or L-cell cultures, it was found that S_2 and L_1 direct synthesis of the same size classes of RNA, i.e., 12 to 15*S*, 28*S*, and 38*S*. The major difference between L_1 or S_2 infections in this regard was in the proportion of each size class of RNA produced. L_1 always synthesized

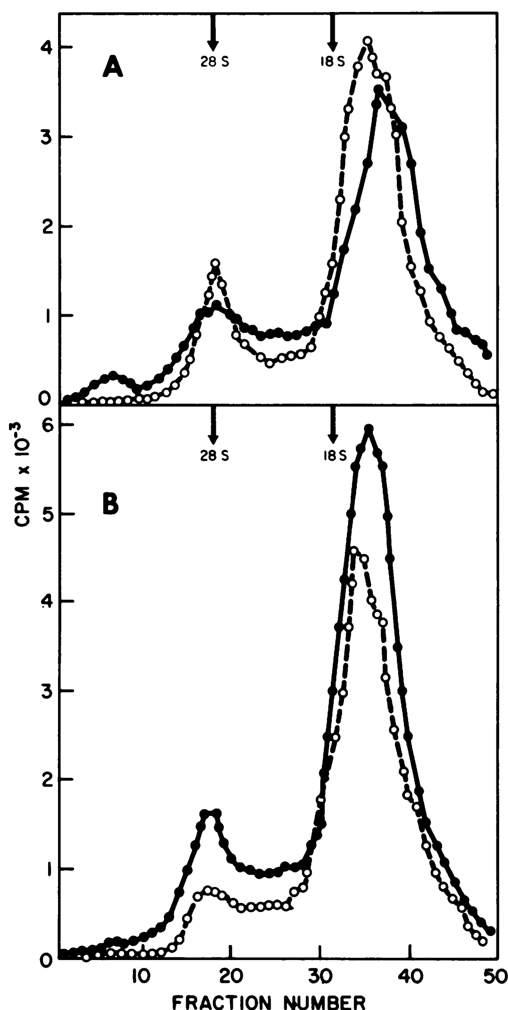

FIG. 8. *Velocity sedimentation analysis of S_2VSV RNA synthesized in CE cells in the absence (●) or presence (○) of cycloheximide (50 µg/ml). Duplicate sets of actinomycin D-treated CE cells were infected with S_2 at a multiplicity of 3. At 3 (a) or 4 (b) h postinfection cycloheximide was added to one set of cultures. Thirty minutes after addition of cycloheximide both sets of cultures were exposed for 60 min to 4 µCi of ^{3}H-uridine. At the end of the labeling period, cytoplasmic extracts were prepared, made 1% with SDS, and analyzed by centrifugation in sucrose gradients for radioactivity in acid-insoluble material.*

larger proportions of 38S RNA, and, in contrast, S_2 produced greater proportions of 12 to 15S material (Table 1 and Fig. 1). Because the RNA in S_2 and L_1 particles is 38S, these findings may indicate that the poor viral yield of S_2 compared with L_1 is due to the failure of S_2 to produce adequate amounts of this class of RNA.

Our results confirm the finding (9) that 38S RNA is not produced unless protein synthesis occurs after VSV infection. The enzymatic machinery brought into the cell by the virion is sufficient to produce only 12 to 15S and 28S RNA (Fig. 5), which is complementary to virion RNA (9). Production of 38S RNA appears to require the synthesis of new protein(s) or at least the modification of pre-existing molecules. The data reported here extend these findings. First, the results of the experiments showing the effect of cycloheximide on rate of RNA synthesis suggest that there are two phases in the synthesis of viral RNA, an early and a late phase. The early phase extends through the first 1.5 to 2 h postinfection. During this period there seems to be little difference in L_1 or S_2 infections, especially with regard to the effect of cycloheximide on rate of RNA synthesis. Cycloheximide prevented any further increase in the rate of RNA synthesis as seen in untreated controls. At times after 2 h, however, there are marked differences in effect of protein synthesis inhibition on L_1 and S_2 RNA synthesis. The rate of RNA synthesis in L_1 infections was enhanced by cycloheximide-induced inhibition of protein synthesis, whereas in S_2 infections the rate of RNA synthesis remained constant or declined after addition of the drug. Secondly, our data demonstrate that production of 38S RNA requires continuous protein synthesis. Addition of cycloheximide to S_2- or L_1-infected CE or L cells that had already begun to synthesize 38S RNA resulted in cessation of 38S RNA production within 30 min after addition of the drug. Concomitant with the shutoff of 38S RNA formation in L_1-infected cells was a striking increase in synthesis of 12 to 15S and 28S RNA. Inhibition of protein synthesis in S_2 infections also caused cessation of 38S RNA production; however, in contrast to the findings with L_1, no increase in synthesis of 12 to 15S or 28S RNA occurred. These observations will be discussed in reference to several hypotheses concerning the mechanism of VSV RNA replication.

One hypothesis is that, after infection, the input 38S RNA is transcribed to 12 to 15S and 28S messenger RNA. The input 38S RNA would also serve as template for the synthesis of a complementary 38S molecule, which would in turn serve as the template for the replication of progeny RNA. This mechanism of replication might require, at the least, the alteration of the pre-existing virion transcriptase to allow synthesis of a complementary 38S template and/or the synthesis of a new enzyme, an RNA replicase, capable of replicating progeny virion RNA from this template.

A second class of models would suppose, as above, that the input 38S RNA is transcribed to complementary 12 to 15S and 28S RNA but that no complementary 38S RNA is made by using the input 38S RNA as template. Rather, a 38S complementary template could be formed by ligation of small RNA molecules which would then serve as template for 38S RNA replication. A second form of this model suggests that progeny 38S RNA would result from replication of the 28S and smaller RNA species by using the small complementary molecules as template. These products would then be ligated to form progeny 38S particle RNA.

Our present data cannot distinguish between the models described above for 38S RNA formation. However, our observations confirm that protein synthesis must occur after infection in order to have any 38S RNA production, and they also show that there must be continued protein synthesis in order to have continued 38S RNA formation. The increased production (or perhaps accumulation) of 12 to 15S and 28S RNA when protein and, concomitantly, 38S RNA synthesis is inhibited in L_1 infections may be interpreted in a number of ways.

(i) If, as suggested in the second model, 38S RNA formation occurs via a ligation step (to make either template or virion nucleic acid), then shutoff of protein synthesis might cause shutoff of ligase production, and therefore small molecules would be expected to accumulate. An extension of this supposition which is also applicable to the first model arises from the finding that 38S RNA formation stops within 30 min after protein synthesis inhibition. This observation indicates that the new protein required for 38S RNA formation is either unstable or is used up soon after its synthesis. This may suggest that the putative ligase or replicase becomes a part of the VS virion, which could account for its short activity span. The protein may function, be packaged, and leave the cell as a part of the virion, or it may function only after it has become a part of the virion.

(ii) An alternative explanation for the increased production of 28S and 12 to 15S RNA after inhibition of protein synthesis may be the elimination of competition for template. When

the protein(s) necessary for 38*S* RNA production is no longer being made, template would be available only for transcription, which would then occur in the presence of cycloheximide (9, 12; Fig. 5) at an increased rate. This possibility might occur under either model for replication.

Kiley and Wagner (11) have suggested that formation of 38*S* RNA may occur via a process involving RNA ligation. They envisioned ligation of the 23*S* and 28*S* RNAs present in nucleocapsids. We, however, do not observe a peak of RNA in the 23*S* region of gradients of cell extracts infected at the low multiplicity of infection used. Only in infections at multiplicities of approximately 35 or higher do we observe a definite peak of RNA sedimenting at 21*S*. This is the approximate sedimentation coefficient of T particle RNA in our system (Wertz and Levine, unpublished data).

Finally, there is the observation in S_2 infections that although 38*S* formation halts after cycloheximide addition just as in L_1 infections, no increase in 28*S* and 12 to 15*S* production occurs. Rather, 28*S* and 12 to 15*S* synthesis proceeds at the rate achieved before addition of the drug, or declines slightly. Because it is apparent that S_2 can make 38*S* RNA and indeed can synthesize greater total amounts of RNA than can L_1, we must conclude that there is no absolute defect in the S_2 replication process. As previously noted, there appears to be an early and a late phase in S_2 and L_1 RNA synthesis. S_2 and L_1 behave similarly in the early phase but not the late. If we suppose that the early phase involves predominantly transcriptive functions and the late phase encompasses a switch to RNA replication, then it could be that S_2 has a greater affinity for the transcriptive process than the replicative process.

Clearly, the mechanism of VSV replication is not yet resolved. We are currently investigating (i) the complementarity of the RNA species made in the absence and presence of cycloheximide, (ii) the possibility that the 28*S* and smaller RNA species are precursors to 38*S* RNA, and (iii) the appearance of new proteins which correlate with the effects of cycloheximide treatment. We hope that more information on these points will help delineate the mechanism of VSV replication.

ADDENDUM IN PROOF

We have examined the labeled RNA, which is synthesized in increased amounts after addition of cycloheximide, by annealing to excess unlabeled 30*S* viral RNA. Both the 28*S* and 12 to 15*S* RNA species are almost completely complementary to VSV 38*S* (−) strand RNA. This finding indicates that the increase in 28*S* and 12 to 15*S* RNA species after addition of cycloheximide late in infection is an increase in transcription, probably due to availability of additional new template.

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