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Replication of Measles Virus: Continued Synthesis of Nucleocapsid RNA and Increased Synthesis of mRNA in the Presence of Cycloheximide

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The effect of cycloheximide on virus specific RNA synthesis in Vero cells infected with either wild-strain (Edmonston) or subacute sclerosing panencephalitis strain measles virus was investigated. At 3 days postinfection, cells treated with cycloheximide (2.6×10^{-4} M) and then exposed to [^{3}H]uridine showed a marked increase in labeled virus-specific RNA. A major portion of this incremental labeled RNA was putative viral mRNA which sedimented at 16, 22, and 30*S*. Five distinct classes of polyribosomes, which were not evident in untreated cells, were found in cycloheximide-treated cells and each contained similar species of virus-specific RNA. Viral nucleocapsid RNA, 50 and 18*S*, was synthesized and encapsidated in the presence of cycloheximide. The latter observation is in apparent contrast to reports that cycloheximide inhibits replication of RNA of classical paramyxoviruses, and may indicate that mechanisms for replicating RNA of measles virus are different from those for replicating RNA of paramyxoviruses.

Viruses which appear to be variants of classical measles virus have been isolated from subacute sclerosing panencephalitis (SSPE) (8). In attempts to clarify the role of these and other strains of measles virus in the pathogenesis of SSPE, virus-specific components of cells infected with these viruses are being characterized. In a previous study (5), we found that measles virus-infected cells may produce not ony 200*S* nucleocapsids containing 50*S* RNA, the putative viral genome, but also distinct classes of short nucleocapsids containing less than viral genome-equivalents of RNA. In that study, polyribosomes were not prominent in cytoplasmic extracts of infected or noninfected cells. The present report is concerned with synthesis of virus-specific RNA and accumulation of polyribosomes in cells which were treated with cycloheximide after infection.

MATERIALS AND METHODS

Virus, cells, and media. Virus, cells, and media have been described in a previous communication (5). Vero cells were grown in Eagle minimum essential medium (MEM) containing 10% fetal calf serum (FCS). BSC-1 cells were grown in medium 199 containing 20% FCS and 0.1% yeast extract. Wild-strain Edmonston measles virus, originally obtained from H. M. Meyer, Jr., and SSPE-strain virus, MUN-HT, isolated as described previously (8), were propagated in BSC-1 cells.

Labeling infected cells. In a typical experiment, Vero cell monolayers in 8-oz. prescription bottles were infected at a multiplicity of 0.05 PFU per cell. After a 1-h adsorption period, 10 ml of MEM containing 2% FCS was added to each culture and cultures were incubated at 37 C in 5% CO_2. At a time noted for each experiment, cells were exposed to 25 μg of actinomycin D (Calbiochem) per ml and 2 h later they were exposed to 20 μCi of [5-^{3}H]uridine per ml (28 Ci/mmol, Schwarz/Mann). When indicated, cycloheximide (Sigma) was added to a final concentration of 75 μg/ml (2.6×10^{-4} M) 1 h prior to labeling. At the end of the labeling period, cells were harvested and a cytoplasmic extract was prepared. Mock-infected cells served as controls.

When proteins were to be labeled, Vero cell monolayers in 60-mm plastic petri dishes were infected or mock-infected and then incubated with MEM containing 2% FCS. Actinomycin D and/or cycloheximide were added to some cultures at 3 days postinfection. Two hours after actinomycin D and 1 h after cycloheximide was added, the medium was replaced with MEM-2% FCS containing 5% of the regular amino acid concentration and 5 μCi of tritium-labeled reconstituted protein hydrolysate per ml (Schwarz/Mann) in addition to actinomycin D and cycloheximide. After a 3-h labeling period, the monolayers were washed twice with 5 ml of ice cold Tris-buffered saline (TBS: 0.01 M Tris, 0.14 M NaCl, pH 7.4). Cells were scraped into 5 ml of cold TBS, pelleted at $1,500 \times g$,

and twice precipitated with 5% cold trichloroacetic acid. The final precipitate was dissolved in 0.3 ml of NCS:toluene (7:3) for liquid scintillation counting.

Cell fractionation. Cytoplasmic extracts were prepared essentially by the method of Penman et al. (9). After medium was removed from cultures, the cells were scraped into 5 ml of TBS. Cells were pelleted at 1,500 × g for 10 min at 4 C and resuspended in 1 to 3 ml of reticulocyte standard buffer (RSB:0.01 M Tris, 0.01 M NaCl, 0.0015 M $MgCl_2$, pH 7.4) and allowed to swell for 20 min in an ice bath. Cells were disrupted with 15 strokes of a Dounce homogenizer and the cytoplasmic extract decanted after nuclei were pelleted at 800 × g for 10 min at 4 C.

After the extracts were made 0.5% with respect to the detergents deoxycholate (Sigma) and BRIJ-58 (Atlas Biochemical Industries), analysis was carried out by centrifugation through 15 to 40% (wt/vol) 38-ml sucrose gradients prepared in RSB. Centrifugation was for 3 to 3.5 h at 95,000 × g at 4 C using an SW27 rotor. Gradients were fractionated into 1.2-ml samples employing an ISCO density gradient fractionator (ISCO, Lincoln, Neb.) while absorbance at 260 nm was continuously monitored. Some extracts were made 0.02 M with respect to EDTA and analyzed on 15 to 40% sucrose gradients prepared in RSB-EDTA buffer (0.01 M Tris, 0.01 M NaCl, 0.03 M EDTA, pH 7.4).

Extraction and analysis of RNA. Extraction and analysis of RNA from cytoplasmic extracts and sucrose gradient fractions were as described previously (6).

RESULTS

Effect of cycloheximide on macromolecular synthesis. To determine the effect of cycloheximide on protein and RNA synthesis, infected and uninfected cells were labeled with radioactive amino acids or uridine after treatment with cycloheximide. Since actinomycin D was to be used to inhibit cellular RNA synthesis so that virus-specific RNA synthesis could be measured, the effects of cycloheximide were examined in cultures in the absence and in the presence of actinomycin. Cells were harvested after incubation with the radioactive precursors and acid insoluble radioactivity determined. As shown in Table 1, cycloheximide (75 μg/ml) reduced protein synthesis by greater than 98% in both uninfected and infected cells. Actinomycin D had relatively little effect on protein synthesis.

The effect of cycloheximide on viral RNA synthesis was determined in the presence of a concentration of actinomycin D which was previously shown to reduce cellular RNA synthesis by 99% (5). Data in Table 2 show that the effect of cycloheximide on actinomycin-D-resistant RNA of uninfected cells was slight, whereas its effect on that of infected cells was marked. In cells infected with wild-strain virus, cycloheximide caused an increase of about 300% in labeled RNA. In cells infected with SSPE virus this increase was about 170%.

TABLE 1. *Effect of cycloheximide on protein synthesis of measles virus-infected cells*[a]

Cell-source	Cycloheximide added (75 μg/ml)	Actinomycin D added (25 μg/ml)	Acid-insoluble radioactivity (counts/min)
Uninfected	No	No	160,000
	No	Yes	147,000
	Yes	No	1,500
	Yes	Yes	1,760
Infected (wild strain)	No	No	140,000
	No	Yes	163,000
	Yes	No	1,480
	Yes	Yes	1,780
Infected (SSPE strain)	No	No	122,000
	No	Yes	139,000
	Yes	No	1,190
	Yes	Yes	1,440

[a] At 3 days postinfection, cells were exposed to [^{3}H]amino acids for 3 h before harvest. Where indicated, cycloheximide and actinomycin D were added, 2 and 1 h, respectively, before exposure to labeled amino acids.

TABLE 2. *Effect of cycloheximide on RNA synthesis of measles virus-infected cells*[a]

Cell-source of cytoplasmic extract	Counts per min per OD_{260} — Cycloheximide −	Counts per min per OD_{260} — Cycloheximide +	Increase with cycloheximide (%)
Uninfected	750	840	12
Infected (wild strain)	3,900	15,000	300
Infected (SSPE strain)	1,700	4,600	170

[a] At 3 days postinfection, all cultures were exposed to [^{3}H]uridine for 6 h before harvest. Actinomycin D was added to all cultures at 2 h and cycloheximide was added where indicated at 1 h before addition of [^{3}H]uridine. RNA was extracted from extracts with SDS-phenol, precipitated with an equal volume of cold ethanol, and resuspended in 0.5 ml of NTE (0.1 M NaCl, 1 mM EDTA, and 0.01 M Tris, pH 7.4) containing 0.01% SDS. Absorbency at 260 nm was determined. A portion with added BSA was twice precipitated in ice cold 5% trichloroacetic acid and radioactivity in the final precipitate was measured by scintillation spectroscopy.

RNA species produced in the presence of cycloheximide. The results described above

demonstrated an increase in labeled virus-specific RNA in the presence of cycloheximide. This virus-specific RNA was then characterized. At 3 days postinfection, cultures were treated with actinomycin D and exposed to [^{3}H] uridine in the presence or absence of cycloheximide. RNA was isolated from cytoplasmic extracts and analyzed by sedimentation in 5 to 20% sucrose gradients. Figure 1 illustrates gradient patterns of RNA extracted from cultures infected with wild- or SSPE-strain virus and labeled with [^{3}H]uridine in the absence or presence of cycloheximide. Four species of RNA with sedimentation coefficients of approximately 16, 22, 30, and 50*S* were obtained from cells infected with wild-strain virus regardless of whether the RNA was synthesized in the absence (Fig. 1A) or presence (Fig. 1B) of cycloheximide. Cycloheximide treatment produced marked increases in the amounts of 16, 22, and 30*S* RNA and a small decrease in the amount of 50*S* RNA. Cycloheximide treatment of cells infected with SSPE-strain produced similar results (Fig. 1C and D). Fractions from 50*S* regions of these gradients (Fig. 1A to D) contained less than 150 counts/min. Thus, the increased labeling of virus-specific RNA observed in the presence of cycloheximide was attributable to increased amounts of label in the smaller species of RNA. In our experience, amounts of 50*S* RNA present in sodium dodecyl sulfate (SDS)-phenol extracts of measles-infected cells have been small and poorly reproducible. For this reason, we do not know if the apparent decrease in labeling of extracted 50*S* RNA, which was seen after cycloheximide treatment, is significant.

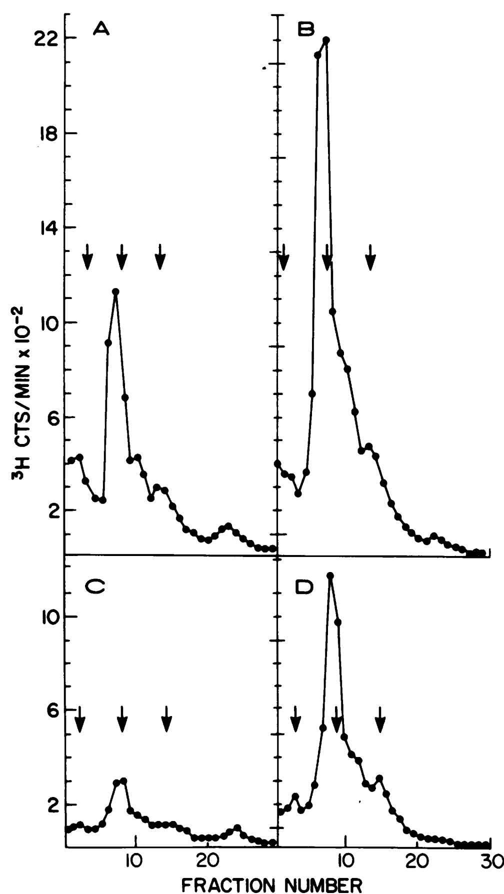

FIG. 1. *Effect of cycloheximide on viral RNA synthesis. At 3 days postinfection, cells were treated with actinomycin D and 1 h later with cycloheximide. After 1 h in the presence of cycloheximide, cells were exposed to [^{3}H]uridine for 6 h, harvested, and a ctyoplasmic extract was prepared. RNA was extracted by SDS-phenol and analyzed on 17-ml 5 to 20% (wt/vol) sucrose gradients prepared in 0.1 M acetate buffer. Panels represent the RNA patterns from (A) wild-strain-infected cells not treated with cycloheximide, (B) wild-strain-infected cells treated with cycloheximide, (C) SSPE-infected cells not treated with cycloheximide, and (D) SSPE-infected cells treated with cycloheximide. Centrifugation was for 13 h at 23,000 rpm and 4 C in an SW27 rotor. [^{14}C]Vero cell RNA was added as marker. Sedimentation is from left to right and the arrows represent the position of 4, 18, and 28S marker RNA in the gradient.*

Location of virus-specific RNA in subcellular structures. To localize the RNA found in cycloheximide-treated cells, cytoplasmic extracts of drug-treated and untreated, infected and uninfected cells were analyzed on 15 to 40% sucrose gradients. Figure 2 depicts optical density profiles of representative gradients. Extracts from untreated cultures, whether infected with wild strain or SSPE-strain or uninfected, contained 40, 60, and 80*S* ribosome monomer. When extracts from cycloheximide-treated infected and uninfected cells were analyzed, material which appeared to represent polyribosomes was also detected.

Cells, 3 days after infection with wild-strain virus, were then exposed to [^{3}H]uridine in the presence of actinomycin D and cycloheximide. A cytoplasmic extract was prepared and divided in two portions. One portion was analyzed on a 15 to 40% sucrose gradient in RSB while the other was treated with EDTA and analyzed on a 15 to 40% sucrose gradient containing EDTA. The results are presented in Fig. 3 where it can be seen that the virus-specific RNA was present in five distinct peaks heavier than 80*S* in the sucrose gradient in RSB. These peaks were attributed to polyribosomes since their associated RNA was ribonuclease sensitive and their

RNA was released by EDTA treatment. The ribonuclease-resistant structures which were present in the regions around fractions 10 and 18 in both gradients were viral nucleocapsids which we have described earlier (5). These observations indicated that cycloheximide treatment resulted in an accumulation of polyribosomes which in actinomcyin-D-treated infected cells contained viral mRNA. This mRNA appeared to account for much of the increased labeled RNA after cycloheximide treatment. The presence of [^{3}H]uridine-labeled nucleocap-

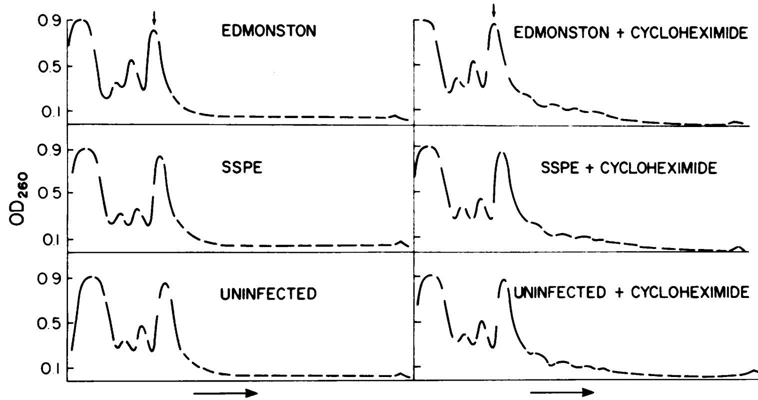

FIG. 2. *Polyribosome accumulation in the presence of cycloheximide. At 3 days postinfection, actinomycin D was added to each culture and, where indicated, cycloheximide was added 1 h later. Two hours after actinomycin D was added, cells were harvested. Detergent-treated cytoplasmic extracts were analyzed in 15 to 40% (wt/vol) sucrose gradients, prepared in RS buffer. Shown are optical density tracings from gradients containing extracts from cells infected with wild or SSPE strain virus or from uninfected cells. Sedimentation is from left to right and the arrow marks the peak of 80S ribosome monomer in each gradient.*

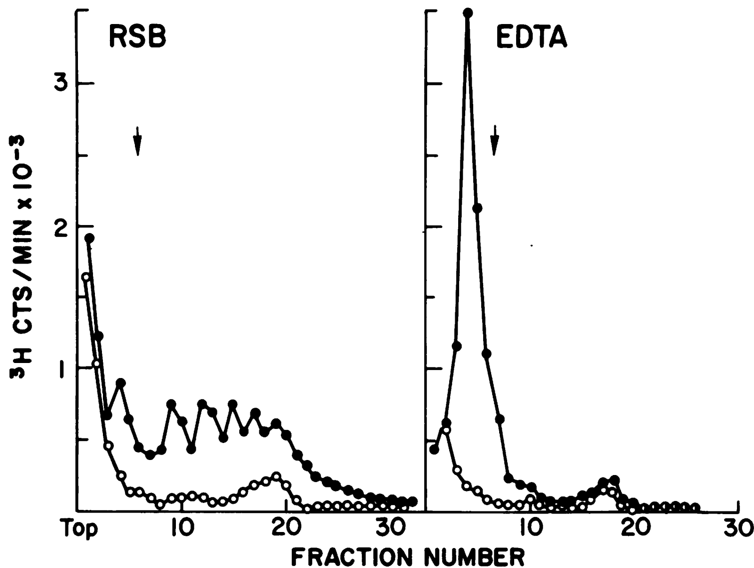

FIG. 3. *Association of virus-specific RNA with polyribosomes in cycloheximide treated cells. At 3 days postinfection cells were labeled for 5 h with [^{3}H]uridine in the presence of actinomycin D and cycloheximide as described for Fig. 1. A detergent-treated cytoplasmic extract was divided into two equal portions. One portion was layered onto a 15 to 40% sucrose gradient prepared in RSB buffer, whereas the other was first treated with 0.1 volume of 0.2 M EDTA before layering on a 15 to 40% sucrose gradient prepared in RSB-EDTA buffer. The distribution of trichloroacetic acid-precipatable radioactivity before (●) and after (○) ribonuclease treatment as shown for each gradient. Sedimentation is from left to right and the arrow represents the position of the 80S monomer in the RSB gradient and the 50S monomer in the EDTA gradient.*

sids indicated that their associated RNA had been synthesized in the presence of cycloheximide.

RNA from cycloheximide-induced polyribosomes. Since labeling RNA in the presence of cycloheximide resulted in the appearance of five distinct polyribosome classes, we wished to determine the type of RNA associated with each class. This was accomplished by labeling virus-specific RNA in the presence of cycloheximide, separating the polyribosomes by sucrose gradient, and analyzing the RNA from each polyribosome peak by velocity sedimentation analysis in sucrose. The results of such an experiment are presented in Fig. 4. In Fig. 4A, the effect of cycloheximide is manifested by the five polyribosome peaks. In the absence of the drug only nucleocapsid structures, 200*S* and a small amount of 110*S*, were apparent. Figure 4B through F represents the RNA species from each of the five polyribosome peaks from the lightest to the heaviest, respectively. RNA from each polyribosome peak sedimented with a peak in the 16*S* region and a shoulder in the 22*S* region of the gradient. It appeared as though each polyribosome peak contained a similar population of RNA molecules. From the gradient containing the extract of cycloheximide-treated cultures, the 200*S* region yielded, in addition to 16*S* RNA, 50*S* RNA (Fig. 4F) which was also present in the 200*S* nucleocapsids from cultures not treated with cycloheximide (Fig. 4G). This observation suggested that 50*S* virion RNA had been synthesized and encapsidated in the presence of cycloheximide.

Effect of cycloheximide on nucleocapsid synthesis. Results presented in Fig. 3 indicated that the RNA of viral nucleocapsids was labeled in the presence of cycloheximide. Previous studies have indicated that this drug inhibits synthesis of virion (50*S*) RNA in cells infected with Sendai virus (11, 14), Newcastle disease virus (1, 4), or mumps virus (2) and turns off synthesis of the virion RNA (38*S*) in vesicular stomatitis virus-infected cells (13; M. P. Kiley, unpublished observation). To directly test the effect of cycloheximide on the production of measles virus nucleocapsid RNA, replicate cultures were infected with wild-strain virus and were labeled 3 days later with [^{3}H]uridine in the presence of actinomycin D and either in the absence or in the presence of cycloheximide. Cytoplasmic extracts were prepared and analyzed in 15 to 40% sucrose gradients in RSB. The results of a representative experiment are presented in Fig. 5. In the gradient of extract from cultures not treated with cycloheximide (5A), two major nucleocapsid populations, with sedimentation coefficients of 110 and 200*S*, respectively, were readily distinguished. In the gradient of extract from the cycloheximide-treated cultures (5B), the five distinct polyribosomal peaks were again

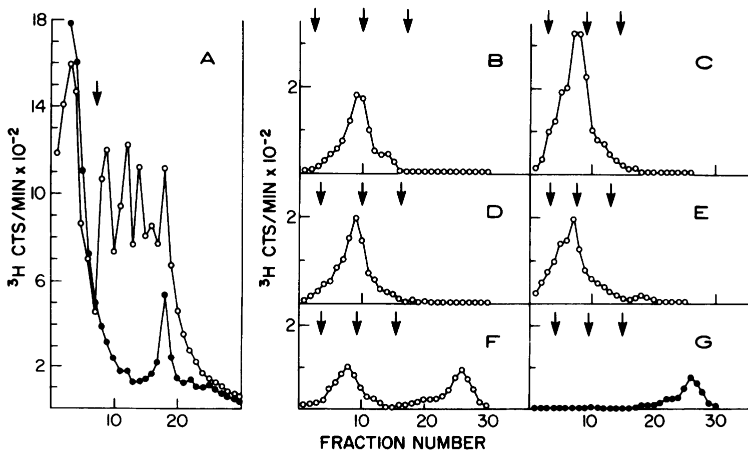

FIG. 4. *Virus-specific RNA present in cycloheximide-induced polyribosomes. Wild-strain-infected cells were labeled at 3 days with [^{3}H]uridine in the presence of actinomycin D, with or without exposure to cycloheximide, as described for Fig. 1. Part A represents analysis of extracts, from cells either treated* (O) *or not treated* (●) *with cycloheximide, on 15 to 40% sucrose gradients in RSB. RNA was extracted from peak fractions of A by SDS-phenol and analyzed on 17-ml 5 to 20% sucrose gradients prepared in 0.1 M acetate buffer, pH 5.0. The sucrose gradient profiles of RNA extracted from gradient fractions 9, 12, 14, 16, and 18 from cycloheximide-treated extracts are depicted in B through F, respectively. RNA extracted from fraction 18 of the extract from noncycloheximide-treated cultures depicted in A is presented in G. Sedimentation is from left to right. In A the arrow represents the position of 80S monomer in the gradient. In B through G arrows indicate positions of ^{14}C-labeled 4, 18, and 28S ribosomal marker RNAs.*

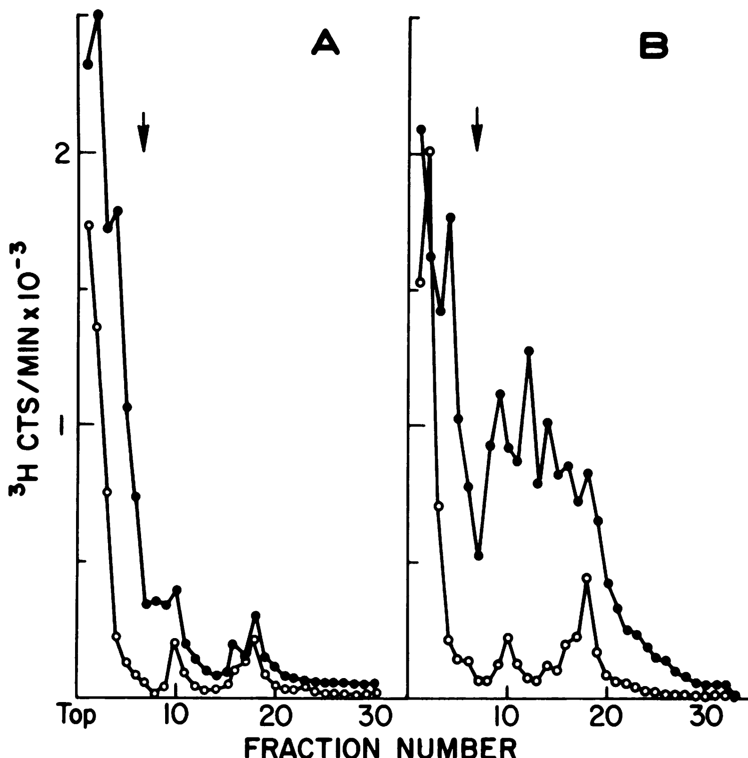

FIG. 5. *Effect of cycloheximide on viral nucleocapsid RNA synthesis. Replicate cultures of wild-strain virus-infected cells were exposed to actinomycin D at 3 days postinfection. One hour later, one set of cultures received cycloheximide and 1 h later both sets were exposed to [^{3}H]uridine. After a 6-h labeling period, detergent-treated cytoplasmic extracts were prepared and analyzed in 15 to 40% sucrose gradients in RSB buffer. Trichloroacetic acid-precipitable radioactivity was determined on portions of each fraction before (●) and after (○) ribonuclease treatment. The profile of an extract from cells not treated with cycloheximide is shown in panel A and that of cycloheximide-treated cells in panel B. Sedimentation is from left to right and the arrow represents the position of 80S ribosomal monomer in the gradient.*

present and the nucleocapsid peaks were apparent after ribonuclease treatment. In both cycloheximide-treated and nontreated cultures, nucleocapsids and their associated RNA were synthesized and there may have been a slight increase in the amount of labeled RNA, presumably 50*S*, in 200*S* nucleocapsids from cycloheximide-treated cells. This method of indirectly measuring labeled 50*S* RNA by its association with nucleocapsids appeared to be more sensitive and reproducible than examining the SDS-phenol-extracted RNA from cytoplasm (i.e., Fig. 1). Under similar conditions this dose of cycloheximide completely suppressed synthesis of nucleocapsid-associated RNA (38*S*) in Vero cells infected with vesicular stomatitis virus (data not shown).

DISCUSSION

Measles virus-infected cells treated with cycloheximide and then exposed to [^{3}H]uridine were found to show a marked increase in labeled virus-specific RNA. A major portion of this incremental labeled RNA appeared to be mRNA associated with polyribosomes which were prominent only in cultures treated with cycloheximide. The observed accumulation of polyribosomes after cycloheximide treatment is in accord with previous reports indicating that this drug, at doses similar to that used in the present experiments, affects slowing of movement of ribosomes along the messenger (3, 12).

The mechanisms involved in the cycloheximide-induced increase in labeled virus-specific mRNA are unknown. Possible effects of cycloheximide on nucleotide pool size have not been determined. To propose that accumulation and stabilization of mRNA in polyribosomes was responsible for the increased amount of labeled RNA would assume that, in the absence of cycloheximide, the virus-specific messenger is quite short-lived. There is no

direct evidence bearing on this assumption. If measles virus messenger is not labile, then an increased rate of transcription must be considered. Because cycloheximide inhibited synthesis of vesicular stomatitis virion RNA, Wertz and Levine (13) suggested that elimination of competition for template RNA was responsible for increased amounts of labeled viral mRNA. This explanation does not seem adequate to explain the effect observed in cycloheximide-treated measles-infected cells because the drug did not inhibit synthesis of measles nucleocapsid RNA. The recent finding that Sendai virion envelope proteins inhibit in vitro transcription of Sendai virion RNA (7) and a similar finding with vesicular stomatitis virus (10) suggest that cycloheximide may inhibit the synthesis or action of an inhibitor, perhaps virion protein, which controls measles virus-specific transcription. Regardless of the mechanisms involved in increasing the amount of labeled mRNA after cycloheximide treatment, the phenomenon should facilitate identification of measles virus-specific messengers, comparison of measles strains by RNA-hybridization, and identification of measles genome products in nonproductive infected cells from SSPE brain.

Our observation that cycloheximide did not decrease the amount of labeled measles nucleocapsid RNA is in apparent contrast with previous reports that protein synthesis is required for synthesis of virion RNA of paramyxoviruses (1, 2, 4, 11, 14) and of vesicular stomatitis virus (13). More specifically, Robinson (11) found that cycloheximide markedly suppressed synthesis of Sendai virus nucleocapsid-associated RNA which was complementary to Sendai viral mRNA. This difference between measles virus and the other negative-strand RNA viruses apparently is not attributable to differences in host-cell systems used in the various studies since cycloheximide (75 μg/ml) was found to inhibit synthesis of vesicular stomatitis nucleocapsid RNA (38*S*) in Vero cells (M. P. Kiley, unpublished observation). Although the observed lack of decrease in amount of label in measles nucleocapsid RNA after cycloheximide treatment of infected cells may relate to polarity of the contained RNA, alteration of nucleotide pool size, or accumulation of cytoplasmic nucleocapsids, it is also possible that the RNA-replication enzyme of measles virus is unusually stable. It is not known whether a fundamental difference exists between the mechanism for replicating RNA of measles virus and that for replicating RNA of the classical paramyxoviruses. However, the fact that cycloheximide did not suppress the amount of label found in measles nucleocapsid RNA suggests that the possibility of such a difference should be considered.

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