THE REGULATION OF RNA SYNTHESIS AND PROCESSING IN THE NUCLEOLUS DURING INHIBITION OF PROTEIN SYNTHESIS

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ABSTRACT

The effect of protein synthesis inhibition by cycloheximide on nucleolar RNA synthesis and processing has been studied in HeLa cells. Synthesis of 45S RNA precursor falls rapidly after administration of the drug. However, the nucleolar content of 45S RNA remains relatively constant for at least 1 hr because the time required for cleavage of the precursor molecule into its products is lengthened after treatment with cycloheximide. The efficiency of transformation of 45S RNA to 32S RNA remains constant with approximately one molecule of the 32S RNA produced for each cleavage of a molecule of 45S RNA. However, shortly after the cessation of protein synthesis the formation of 18S RNA becomes abortive. The amount of 32S RNA present in the nucleolus remains relatively constant. After long periods of protein synthesis inhibition the 28S RNA continues to be synthesized and exported to the cytoplasm but at a greatly reduced rate. When the protein synthesis inhibitor is removed, a prompt, although partial, recovery in the synthesis rate of 45S RNA occurs. The various aspects of RNA synthesis regulation and processing are discussed.

INTRODUCTION

The nucleolus is a highly structured and specialized organelle. Its principal and perhaps only function is the production of ribosomes (1–4). The regulation of ribosomal precursor RNA synthesis and processing under conditions of protein synthesis inhibition is the subject of this report.

It has been shown previously by Warner that the structural proteins for ribosomes can be found associated with the nucleolus (5). Furthermore, a pool of these structural proteins must exist, since under conditions of protein synthesis inhibition newly labeled ribosomal RNA can be detected in the functioning ribosomes in the cytoplasm (6). Previous studies of nucleolar function after the inhibition of protein synthesis have established the following points. The nucleolus can still produce mature ribosomes, although at a reduced rate (6). However, the formation of 18S RNA from 45S precursor ceases soon after inhibition (7, 8). Since 32S and 28S RNA continue to be formed, it is apparent that 18S RNA is degraded under these conditions. Soeiro, Vaughan, and Darnell have shown that the time for processing 45S ribosomal precursor is increased during the inhibition of protein synthesis (8). They also established that the production of aberrant polypeptides in puromycin-treated cells results in further interference

with nucleolar function so that no cytoplasmic 28S RNA is obtained.

In this report, a detailed study of nucleolar function during the inhibition of cellular protein synthesis is described. The response of the nucleolus appears to be regulated. The rate of 45S ribosomal precursor falls rapidly in the presence of the protein synthesis inhibitor, cycloheximide. However, the nucleolar content of 45S and 32S RNA changes only slightly. This decrease in the rate of synthesis without a concomitant decrease in the nucleolar content of RNA is shown to be the result of an increased length of time required for RNA processing. Thus, synthesis and processing time are interrelated in such a way as to maintain a relatively constant nucleolar content of RNA.

Even though nucleolar function is drastically altered during protein synthesis inhibition, a constant efficiency of transformation of 45S to 32S is maintained. However, as shown previously, production of 18S RNA rapidly ceases after protein synthesis inhibition. Presumably, the inhibition of synthesis of structural proteins results in this species being degraded after it is cleaved from the 45S precursor.

MATERIALS AND METHODS

The experiments were performed with suspension cultures of S-3 HeLa cells growing in Eagle's medium supplemented with 7% horse serum at a nominal concentration of 4×10^5 cells/ml (9). Cycloheximide was used at a concentration of 100 μg/ml, except when specified. Before addition to the culture medium of radioactive RNA precursors, the cells were concentrated to 2×10^6 cells/ml. The preparation of nucleolar, nucleoplasmic, and cytoplasmic fractions was as described (2) except that the nucleoli were separated from DNase-digested nuclei by zonal centrifugation through a sucrose density gradient (10). SDS-phenol extraction of the RNA in the three cellular fractions was as described (11), except that the temperature of extraction was 55°C (12). The purified RNA was analyzed by SDS-sucrose gradients (13) or by acrylamide gel electrophoresis (14, 15). The composition of the buffer used in SDS sucrose gradients was the following: 0.5% sodium lauryl sulfate (SDS), 0.1 M NaCl, 0.01 M Tris, pH 7.4, 0.001 M EDTA.

Cycloheximide (Acti-dione) was purchased from Calbiochem, Los Angeles, Calif. Electrophoretically purified DNase was obtained from Worthington Biochemical Corp., Freehold, N. J.

Actinomycin was a gift of Merck, Sharp & Dohme.

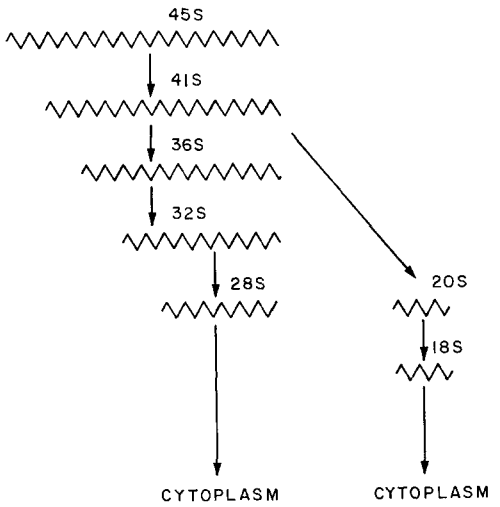

FIGURE 1 Schematic representation of the stages in processing of the 45S RNA nucleolar precursor to ribosomes.

Uridine-2-^{14}C (27 mc/mmole) and uridine-5-^{3}H (20 mc/μmole) were purchased from Schwarz Bio Research Inc., Orangeburg, N. Y.

RESULTS

The experimental results presented here can be best understood in terms of results of previous investigations which outlined the details of RNA processing in the nucleolus (15), which are illustrated in Fig. 1. The initial event in the formation of ribosomal RNA is the synthesis and methylation of a larger precursor molecule whose sedimentation is 45S. The precursor remains as 45S for about 16 min and then is cleaved to 18S and 32S RNA. The process of cleaving the precursor molecule to form its products is not conservative because the combined molecular weights of the 18S and 32S species of RNA are much less than the molecular weight of the 45S precursor (10, 15, 16,[1]). Evidence for the loss of material during the normal processing of 45S has been obtained by studying methyl to uridine ratios of the various species of RNA (10, 15), the base compositions of precursors and products (10, 16), and the pancreatic RNase digests of the various species (16). All these techniques point to a disappearance of more than 30% of the 45S molecule during the formation of 18S and 32S

[1] McConkey, E. Private communication.

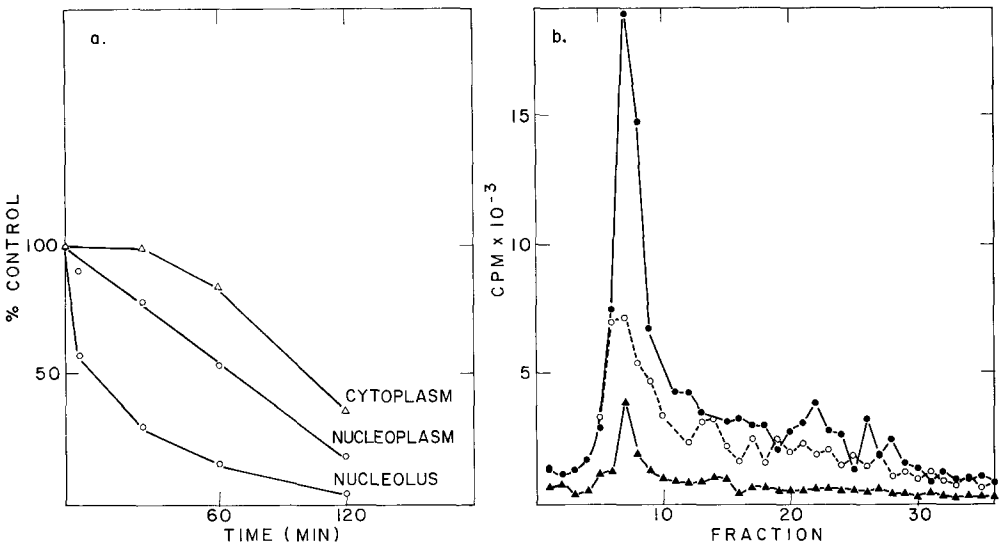

FIGURE 2 10^8 cells were suspended in 50 ml of medium and divided as follows. 2×10^7 cells (control) were labeled for 10 min with uridine-5-^{3}H (5 μc/ml; sp. act. 25 mc/μmole). Incorporation was terminated by pipetting the cells onto crushed frozen Earle's saline. Equal aliquots of cells were treated with cycloheximide for 5, 30, 60, and 120 min, and then labeled as above. Nucleoli were prepared and RNA was extracted as described in Materials and Methods. The cytoplasmic and nucleoplasmic fractions were made 0.5% in SDS (sodium lauryl sulfate) and an aliquot of each was precipitated with 10% trichloroacetic acid, collected on a Millipore filter (Millipore Filter Corp., Bedford, Mass.), and counted in an Ansitron liquid scintillation counter. The extracted nucleolar RNA was analyzed on 2.6% polyacrylamide gels as described in Materials and Methods. Gels were run for 4.5 hr at 5 ma per gel. Optical density (OD) was monitored with a specially adapted Gilford recording spectrophotometer (Gilford Instrument Company, Oberlin, Ohio). The gels were then cut into 1 mm lengths, hydrolyzed with 0.4 ml of NH_4OH, and counted in a scintillation counter. The total radioactivity in the 45S region of the nucleolar gel was calculated and plotted as % control in Fig. 2*a*. Cytoplasmic and nucleoplasmic radioactivity was graphed in the same manner. Fig. 2*b* shows the profile of radioactivity in the nucleolar gels in the control ●—●—● and after treatment with actidione for 5 min —○—○— and 60 min ▲---▲---▲.

products. A clearly observed transient 41S species and a minor 36S species of nucleolar RNA have been identified and are presumably intermediates in the cleavage of the 45S precursor (15).

The 18S molecule is rapidly exported to the cytoplasm where it appears in the small ribosomal subunit. The 32S molecule remains intact in the nucleolus for a minimum of 40 min (2), after which time it is cleaved again to form the 28S species of RNA. This conversion in sedimentation has been demonstrated to be a molecular weight change with approximately 30% of the 32S molecule being lost (10, 15, 16). The evidence that 28S RNA is lower in molecular weight than 32S RNA comes from the study of methyl to uridine ratios of the two species (15), the base composition (10), the comparison of electrophoretic mobility to sedimentation velocity (15), and the pancreatic RNase digest (16).

The 28S RNA cleaved from the 32S remains in the nucleolus or nucleoplasm for another 20 min before emerging into the cytoplasm. An apparent nucleoplasmic stage for this 28S RNA has been previously reported (2, 17), but this may represent, at least in part, RNA which is associated with the nucleolus in vivo and is broken loose by the high-salt treatment used to prepare the nucleolus (18). It should be noted that the lifetimes of the various species of RNA are nominal values and that there is considerable dispersion in the time it takes for individual molecules to transit these stages.

Fig. 2*a* shows the decline in the rate of labeling of various species of HeLa cell RNA after the administration of cycloheximide. Cells were labeled

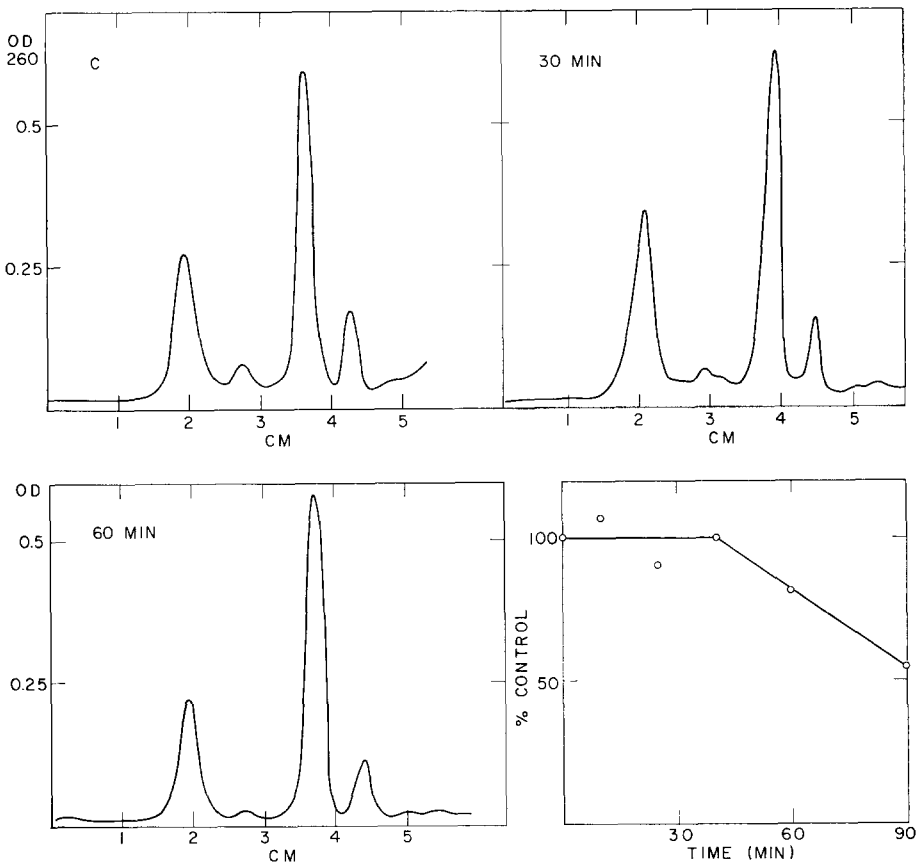

FIGURE 3 1.2×10^8 cells were suspended in 60 ml of medium and labeled for 60 min with uridine-2-^{14}C (0.1 μc/ml; sp. act. 50 mc/mmole) which was diluted with 0.15 mg of unlabeled uridine. An aliquot of 2×10^7 cells was taken (control) and the remaining culture was treated with cycloheximide. Samples were taken at 10, 25, 40, 60, and 90 min after addition of Acti-dione. Nucleoli were prepared and RNA was extracted as described in Materials and Methods. Nucleolar RNA was analyzed on polyacrylamide gels as in Fig. 2. Total radioactivity in the 45S RNA was calculated and plotted as % control.

for 10 min with uridine-^{3}H and fractionated as described in Materials and Methods. The nucleolar RNA labeled in a 10-min pulse consists of 45S RNA and a heterogeneous species of lower molecular weight corresponding, apparently, to partially synthesized chains (19). Some of the decrease in rate of labeling of 45S RNA could be due to an alteration in the equilibration of nucleotide pools with the exogenous label. Although synthesis of all species of RNA declines under these conditions of protein synthesis inhibition, the decrease is much less for both the nucleoplasmic and cytoplasmic species of RNA. Unless the nucleotide pool supplying the ribosomal precursor is distinct from that supplying the other cellular species, it must be concluded that the rate of ribosomal precursor RNA synthesis is rapidly diminished when protein synthesis is inhibited with cycloheximide. No evidence of separate nucleotide pools has yet been observed for mammalian cells.

One additional conclusion can be drawn from the gel electrophoretograms shown in Fig. 2*b*. A diminished rate of synthesis can be due to either fewer polymerase molecules functioning or to a decreased rate of transcription by a constant number of polymerase molecules. If decreased transcription rate were responsible for the inhibition of precursor formation, the ratio of partially synthesized molecules to completed 45S molecules would be increased. No such increase is apparent in Fig. 2*b*, and it is concluded that the

time to complete a 45S molecule remains constant and that fewer polymerase sites are functioning in the inhibited cells.

If synthesis of 45S RNA precursor is inhibited with actinomycin, the amount of 45S RNA in the nucleolus decreases (11). Within 20 min after addition of actinomycin, 45S RNA has nearly disappeared from the nucleolus (2). It might be expected that a decline in the rate of synthesis of 45S RNA during protein synthesis inhibition would be accompanied by a decrease in the amount of this molecule in the nucleolus. However, as shown in Fig. 3, the decline in the rate of synthesis in the presence of cycloheximide is not accompanied by a decrease in the net amount of 45S RNA. 45S RNA content of nucleoli is measured two different ways: First, the optical density of the 45S peak in an acrylamide gel is measured and the area associated with this peak is determined. Alternatively, cells are allowed to incorporate uridine-^{14}C for 1 hr before the administration of cycloheximide. This length of time is sufficient to saturate the 45S species as previously shown (2). Thus, the radioactivity becomes equivalent to the optical density and the amount of RNA labeled in 45S is measured after administering cycloheximide. The results of such an experiment are dependent upon reproducible recoveries between samples. Recoveries are checked with a small aliquot of uridine-^{3}H-labeled cells, which is added to the drug-treated cells before fractionation. The recovery of tritium-labeled 45S RNA should be constant for each sample. The amount of ^{3}H-labeled RNA is measured and used to correct for small differences in recovery. The optical density contributed by the tritium-labeled RNA corresponds to only 2% of the optical density from the drug-treated cells. Thus, measurements of the optical density of 45S RNA are not significantly affected. The results of measuring optical density or radioactivity in 45S RNA agree perfectly.

The RNA present in the nucleolus after inhibition of protein synthesis is shown in Fig. 3. The amount of the major species changes very little. The amount of radioactive 45S RNA present in prelabeled cells after the addition of cycloheximide is also plotted. The nucleolar content of 45S RNA falls very little during the 1st hr of protein synthesis inhibition. In some experiments the amount of 45S actually rises for 30 min before declining slowly.

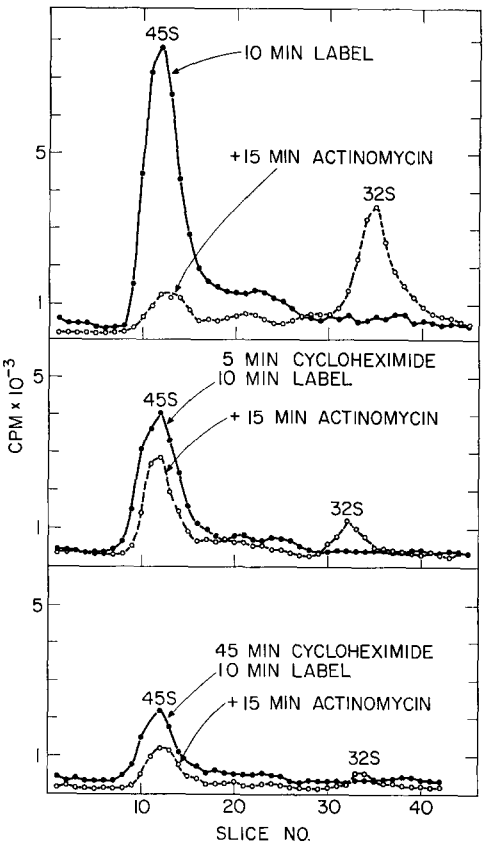

FIGURE 4 4×10^7 cells were suspended in 20 ml of medium and labeled with uridine-5-^{3}H as in Fig. 2. After 10 min, one half of the culture was pipetted onto crushed frozen Earle's saline. Actinomycin D at a concentration of 20 μg/ml was added to the remaining half and the cells were incubated for 15 min more. Two cultures of 4×10^7 cells each were treated with cycloheximide for 5 and 45 min, and then labeled for 10 min and incubated with actinomycin as above. Nucleoli were prepared and RNA was extracted as described in Materials and Methods. RNA was analyzed on polyacrylamide gels as in Fig. 2. —○—○—○ 10-min label; —●—●—● Actinomycin D, 15 min.

Since the rate of synthesis of 45S RNA apparently decreases significantly and the amount of 45S RNA decreases very little, it must be concluded that the rate at which RNA leaves the 45S stage has been reduced. This reduction in the rate of processing is demonstrated directly in the experiments the results of which are shown in Fig. 4. Control cells are labeled with uridine-^{3}H for 10 min and a very high concentration of actinomycin (20 μg/ml) is added to the culture.

TABLE I

Reduction of Rate of RNA Processing during Protein Synthesis Inhibition

	45S	32S	45S remaining after actinomycin	45S decay in 32S
	cpm	*cpm*	%	%
Control	32,304	12,567	15.2	45.9
Control + 15 min. actinomycin	4,923			
15 min cycloheximide	16,971			
15 min cycloheximide + 15 min actinomycin	9,379	2,621	55.3	34.5
55 min cycloheximide	7,511			
55 min cycloheximide + 15 min actinomycin	4,106	1,300	54.7	38.2

Radioactivity in the 45S and 32S peaks in Fig. 4 was estimated. A background was estimated from the heterogeneous RNA migrating to the left of each peak. This background was assumed constant through the peak region and subtracted.

Other cultures are treated with cycloheximide for various lengths of time before labeling and then treated identically. A sample is taken at the same time that actinomycin is added, and a second sample 15 min later. In the control, the labeled 45S RNA has decayed almost completely after 15 min in actinomycin. This agrees with previous measurements of the lifetime of 45S RNA. In contrast, cells which were pretreated with cycloheximide have 55% of labeled RNA remaining as 45S after 15 min in actinomycin. The results are summarized in Table I.

Another important point is illustrated by this experiment. The amount of radioactivity appearing in 32S RNA after the administration of actinomycin without cycloheximide is approximately 40% of the radioactivity which has disappeared from 45S. This is in approximate agreement with previous measurements, which indicate that the molecular weights of 45S and 32S are in the ratio of about 2:1 and that the percentage of uridine is the same in both molecules. The processing of 45S in the presence of actinomycin therefore appears relatively normal. In the presence of cycloheximide, although the rate of transformation is slower, the efficiency of transformation is the same as in untreated cells. Thus, about 40% of the radioactivity disappearing from 45S appears in the 32S peak.

It should be noted that the accuracy of the experiment shown in Fig. 4 is limited by several considerations. First, the result is sensitive to differences in recovery, which alter the apparent amount of 45S RNA that has decayed. In this experiment, the amount of 32S RNA was used to correct for recovery differences, since this slowly processed RNA species changes very little during a 15-min incubation in actinomycin. Another possible source of error is the continued synthesis of 45S RNA for a short time after the administration of actinomycin. Continued synthesis will lead to an underestimate of 45S RNA which has decayed. A high concentration of actinomycin (20 μg/ml) was used in these experiments. Approximately the same results are obtained with 5 μg/ml of actinomycin. It can be concluded that continued synthesis of 45S RNA was probably not an important source of error.

The constant efficiency of transformation applies only to the formation of 32S RNA. The 18S RNA, which is cleaved from the 45S precursor in control cells and rapidly exported to the cytoplasm, is formed in low amounts after the administration of cycloheximide. This is illustrated in the experiment shown in Fig. 5. Cells were labeled for 60 min with radioactive uridine and fractionated as described in Materials and Methods. The nucleolar and cytoplasmic

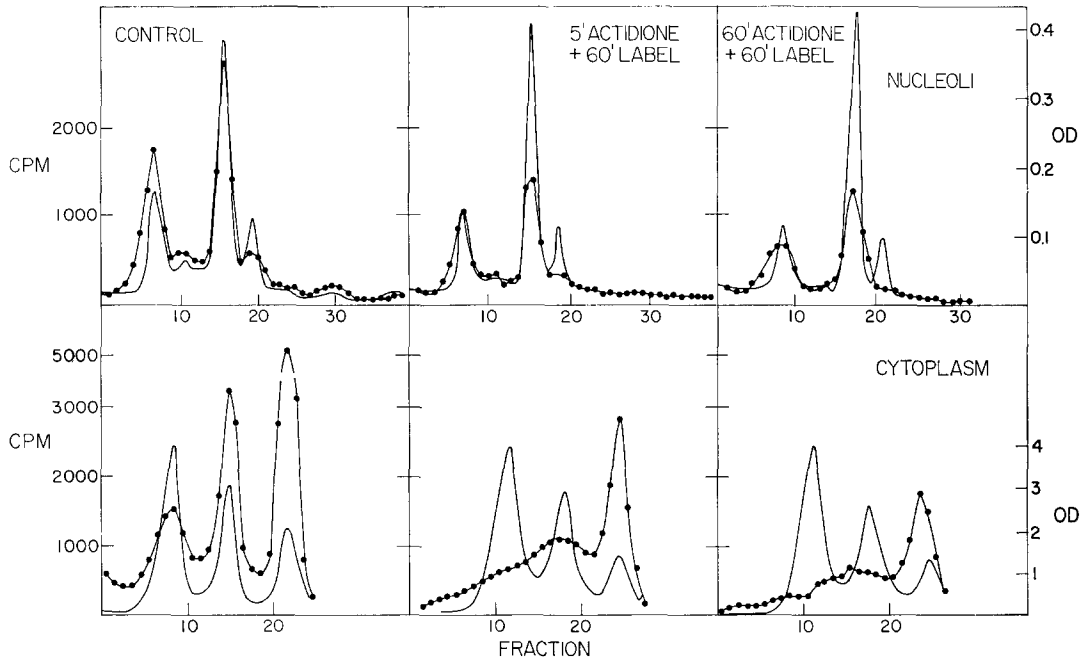

FIGURE 5 2×10^7 cells were suspended in 10 ml of medium and labeled for 60 min with diluted uridine-2-^{14}C as in Fig. 3. Two cultures of 2×10^7 each were treated with cycloheximide for 5 and 60 min, and then labeled as above. Cytoplasm and nucleoli were prepared and the RNA was extracted as described in Materials and Methods. Nucleolar RNA was analyzed on polyacrylamide gels as in Fig. 2. Cytoplasmic RNA was analyzed on SDS sucrose gradients as described in Materials and Methods. Gradients were spun at 25°C for 16 hr at 24,000 rpm in the SW 25.3 rotor of the Beckman L-2 ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.) and eluted through the flow cell of the Gilford recording spectrophotometer. Fractions were collected, precipitated with 10% trichloroacetic acid, and radioactivity was assayed as in Fig. 2. ——— Optical density (OD); —●—●—● Radioactivity.

fractions were extracted and the RNA was analyzed on acrylamide gels for the nucleolus and on sucrose gradients for the cytoplasm. Considerable radioactivity appears in cytoplasmic 18S RNA after 60 min. Previous results, in fact, indicate that the 18S and 32S RNA appear concomitantly (11). In another culture, treated for 5 min with cycloheximide before labeling, there is no radioactivity in cytoplasmic 18S RNA after a 60-min labeling period. Thus, 32S RNA is formed without the concomitant appearance of 18S RNA. It will be shown later that no 18S RNA is present, either in the nucleoli or in the nucleoplasms of these cells. Since there is compelling evidence that both 18S and 32S RNA are contained in a single 45S precursor (20–23), these experiments indicate that the 18S RNA is degraded subsequent to the transformation of 45S to the lower molecular weight products. A similar result has been obtained by other workers (6–8).

It is interesting to note that, despite the declining rate of synthesis of ribosomal precursor and the increased processing time required for 45S RNA, the amount of 32S RNA, as measured by the optical density in this peak, in Fig. 5, remains constant. This suggests that the nucleolus maintains an ordered processing, although at a reduced rate, so that the same number of newly synthesized molecules enter the 32S species as leave, despite the inhibition of new protein synthesis. It is possible that the regulation is due to the maintenance by the nucleolar structure of a constant level of 32S RNA, and that rates of synthesis and processing adjust themselves to keep the amount of 32S RNA relatively constant.

The nucleoli of cells treated for long periods of time with high levels of cycloheximide became

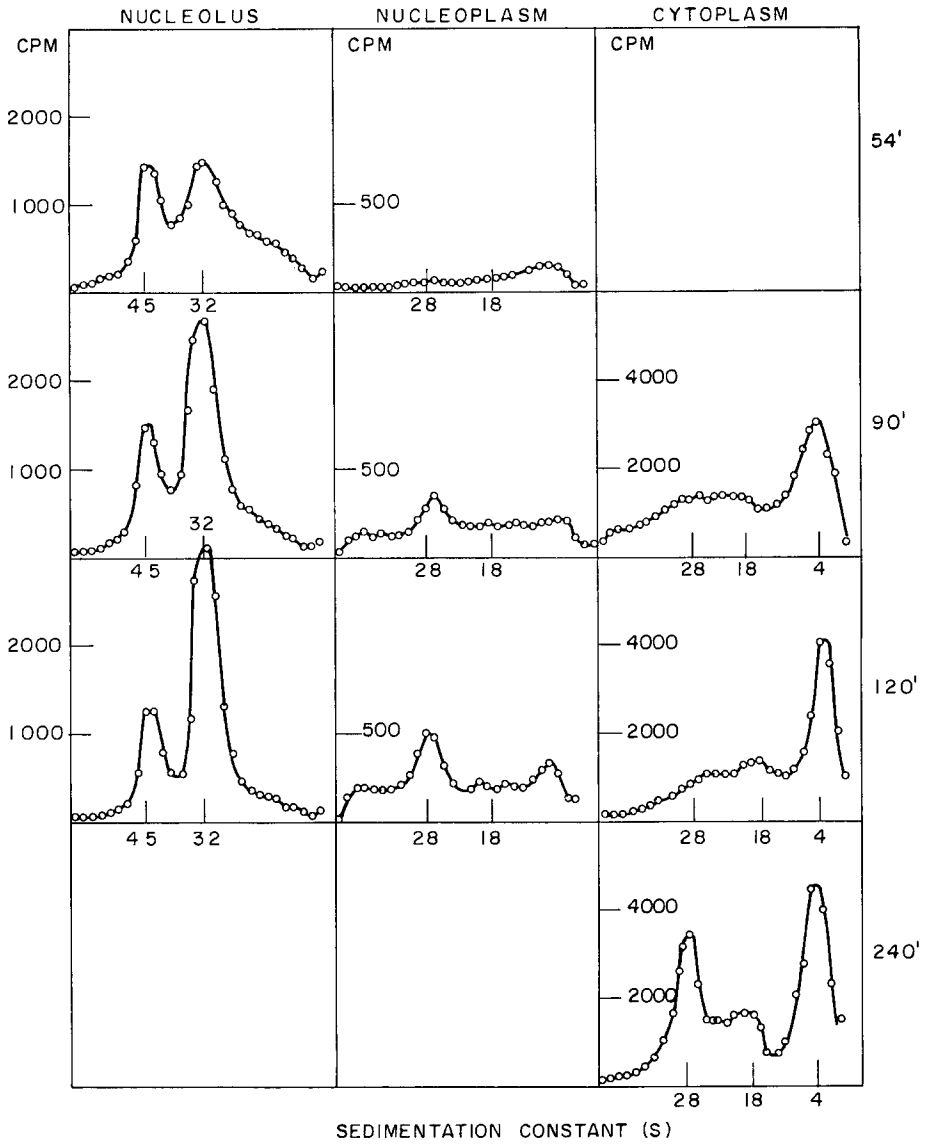

FIGURE 6 2×10^8 cells were suspended in 80 ml of medium and incubated for 30 min with cycloheximide (600 μg/ml) and then labeled with uridine-2-^{14}C as in Fig. 3. Samples of 5×10^7 cells were taken 54, 90, 120, and 240 min later. Cells were fractionated into nucleoli, nucleoplasm, and cytoplasm, and the RNA was extracted as described in Materials and Methods. RNA was analyzed on sucrose density gradients in SDS sucrose buffers as described in Materials and Methods, eluted as in Fig. 5, and the radioactivity was assayed as in Fig. 2. Centrifugation was at 25°C for 16 hr at 21,000 rpm for nucleolar RNA and 16 hr at 24,000 for nucleoplasmic and cytoplasmic RNA in the SW 25.3 rotor of the Beckman L-2 ultracentrifuge.

aberrant in function with greatly reduced levels of RNA synthesis and rates of processing. Nevertheless, even after prolonged periods of protein synthesis inhibition, the nucleolus is able to form mature 28S RNA, which eventually appears in the cytoplasm. In the experiment shown in Fig. 6, HeLa cells were treated with cycloheximide for 1 hr. The greatly reduced rate of processing com-

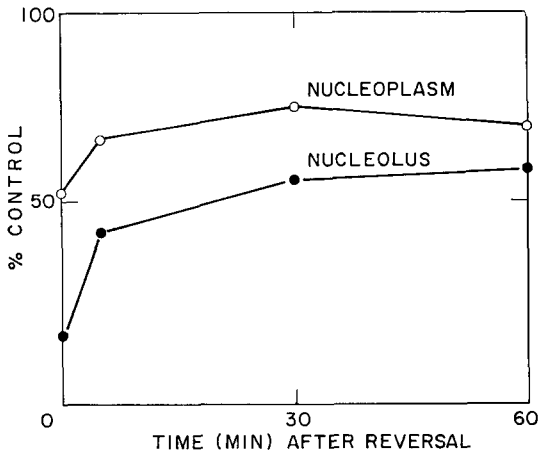

FIGURE 7 10^8 cells were suspended in 50 ml of medium and divided as follows: 2×10^7 cells were labeled for 10 min with uridine-5-^{3}H as in Fig. 2. 8×10^7 cells were incubated with cycloheximide for 60 min. At this time 2×10^7 cells were removed and labeled as above. The remaining cells were washed three times in cold medium, resuspended in warm medium, incubated for 5, 30, and 60 min, and then labeled as above. Nucleoli were prepared and RNA was extracted as described in Materials and Methods. Nucleolar RNA was analyzed on polyacrylamide gels and the total radioactivity in 45S RNA was measured as in Fig. 2. The nucleoplasmic fraction was reserved and an aliquot was measured as in Fig. 2, except that an additional sample was made 0.3 N NaOH and incubated overnight at 37°C. Acid-precipitable radioactivity remaining after this hydrolysis was a measure of incorporation of label into DNA and the samples were corrected by this amount to give total acid-precipitable radioactivity specifically in RNA.

pared to the rate in normal cells can be seen. Some 32S RNA is present after 1 hr of labeling, but an additional 3 hr of incubation is required before a significant amount of 28S RNA emerges into the cytoplasm. This experiment is performed with 600 μg/ml of cycloheximide. At this concentration the rate of protein synthesis is less than 1% of control. It is possible that the meager production of 28S RNA is due to the residual protein synthesis. However, the amount of 28S RNA produced is in excess of 1% of control cells, and the results suggest that a pool of structural proteins exists as previously reported (6). These experiments were performed by úsing sucrose gradients for analyzing all fractions. It is apparent that no 18S RNA is produced under these conditions, which include a long preincubation in cycloheximide.

One further experiment indicates that the decline in nucleolar RNA synthesis is not due to irreversible damage of cellular structure resulting from the presence of the protein synthesis inhibitor. Cells were exposed to cycloheximide for 1 hr and the rate of 45S RNA synthesis was measured. The drug was removed and the recovery of 45S RNA synthesizing capacity was followed. The results are presented graphically in Fig. 7. The rate of 45S synthesis declined to 18% of the value in control cells while the drug was present. After the removal of the drug, synthesis returned to 45% of control within 10 min. The recovery continued, although more slowly, and reached 60% of the control after 60 min. The rapid, although partial, recovery suggests that much of the RNA synthesizing machinery remains intact and is ready to function as soon as new structural proteins become available.

DISCUSSION

The results presented in this report show that the nucleolus of HeLa cells responds promptly to cessation of protein synthesis. Ribosomal RNA synthesis slows drastically. However, the processing time of the 45S RNA precursor also increases so that the RNA content of the nucleolus remains relatively constant.

In normal cells the 45S RNA precursor to ribosomal RNA has a lifetime of about 15 min, after which 18S RNA and a guanosine-cytosine–rich species of RNA are cleaved and removed from the nucleolus, leaving 32S RNA (10, 15, 16). Under conditions of protein synthesis inhibition, the lifetime of 45S RNA is increased and, although 32S RNA is formed from cleavage of the precursor molecule, no 18S RNA appears. However, the production of 32S RNA appears normal in the sense that one molecule of 32S is produced for every molecule of 45S that is cleaved. This is concluded from the results of the decay of 45S RNA in actinomycin. Approximately 40% of the radioactivity which disappears from 45S appears in 32S in both inhibited and control cells. This fraction of radioactivity is predicted on the basis of the molecular weights of the various species.

The increase in processing time measured by actinomycin decay of 45S RNA is consistent with the decrease in the rate of synthesis of 45S RNA without a concomitant decline in the amount of 45S RNA. This increase in processing time has appeared by 15 min after the inhibition of pro-

tein synthesis. However, the increase in processing time does not continue to compensate for the decreased rate of synthesis, and the amount of 45S RNA in the nucleolus begins to decline at about 1 hr.

The normal formation of 32S RNA from its 45S precursor in cycloheximide-treated cells contrasts with results obtained from other methods of interfering with nucleolar function. Results similar to those described here are obtained with puromycin (7). However, the 32S RNA formed is not processed to 28S RNA. This is shown to be a result of the prematurely terminated polypeptides produced by puromycin. During poliovirus infection, 18S RNA is formed normally but 32S RNA formation is greatly suppressed (15, 25). In this latter case there is a buildup of the 41S intermediate, suggesting that the times of processing in normally short-lived states have been greatly increased. The drug cordycepin produces prematurely terminated 45S molecules. Under these conditions some 18S RNA is formed but 32S RNA is suppressed (26).

The function of the nucleolus is clearly regulated in part by the availability of a protein component, which is possibly, but not necessarily, a ribosome structural component. It is possible to speculate about the nature of the signal that regulates synthesis and processing time. One possibility, which is in agreement with results shown here, is that the amount of 32S RNA physically occupying the nucleolus determines the rate of synthesis and processing of 45S RNA. At least the nucleolus functions so as to keep the amount of 32S constant as shown in Fig. 5. Also, the ratio of 45S to 32S labeled during a long incorporation becomes constant as shown by the results in Fig. 6. The ratio of radioactivity in 32S compared to 45S in the cells labeled after a long period of protein synthesis inhibition approaches the ratio found in normal cells. The time necessary to approach this ratio is of course much longer than in normal cells.

Although under conditions of inhibition the nucleolus functions so as to form the 32S successfully and maintain a relatively constant level, the successful formation of 18S RNA from the 45S precursors ceases soon after the inhibition of protein synthesis.

Two models are possible to explain the regulation of 45S RNA synthesis. The results shown in Fig. 2 suggest that decreased synthesis is due to fewer functioning polymerase molecules. Thus, either normally unstable polymerase molecules break down and are not replaced under conditions of protein synthesis inhibition, or polymerase molecules are immobilized and begin to function only when protein synthesis inhibition is reversed. The experiment shown in Fig. 7 indicates rapid recovery in the rate of RNA synthesis after the removal of cycloheximide. This rapid, although partial, recovery suggests that the polymerase molecules exist and can commence function as a new supply of protein for nucleolar processing becomes available. However, experiments of this type cannot determine which of the two models of RNA synthesis regulation is correct.

It may seem odd that nucleolar function is inhibited while a pool of proteins sufficient to finish ribosomes still exists. It appears that the highly organized structure of the nucleolus functions slowly when less than a normal supply of protein is available.

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