JOURNAL OF BACTERIOLOGY, May 1968, p. 1844-1850
Vol. 95, No. 5
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Ribosome Development and the Methylation of Ribosomal Ribonucleic Acid

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Received for publication 23 February 1968

Immature ribonucleoprotein particles accumulate in amino acid-starved cells of a relaxed mutant of *Escherichia coli*. The ribonucleic acid (RNA) of these particles is nonmethylated in cells starved for methionine. However, in bacteria starved for arginine, lysine, or histidine, the RNA of these particles is one-half methylated. The relationship of submethylation to a structural alteration in the same RNA was studied. The results of kinetic studies showed that submethylation and the structural transition are not causally related, since they are described by different rate constants. Moreover, it was possible to accumulate fully methylated immature-particle RNA that possessed the sedimentation and chromatographic properties of nonmethylated RNA. It was concluded that, during the normal course of ribosome development, methylation of ribosomal RNA is completed prior to the final maturation steps.

Ribosomal ribonucleic acid (rRNA) contains measurable quantities of bases which become methylated after incorporation into the polynucleotide (6, 8). The existence of methylated bases in RNA has provided the opportunity for considerable speculation as to their biological significance (11). However, there is still no indication as to the role these nucleotides might play in the biological activity of the nucleic acid.

While investigating steps involved in ribosome development in *Escherichia coli*, Sypherd and Fansler (15) examined the unusual sedimentation and chromatographic properties of rRNA isolated from the immature ribosomes ("relaxed particles") which accumulates when a "relaxed" mutant is deprived of an essential amino acid. Nucleic acid with similar properties accumulates when *E. coli* is treated with chloramphenicol or streptomycin (4, 5). Sypherd and Fansler (15) concluded from their studies that the RNA from immature particles differs from mature ribosomal RNA only in the arrangement of the double-helical regions of the molecules, and not in primary structure of the extent of intramolecular hydrogen bonding. Their studies, however, did not assess the role of polyribonucleotide methylation as it might pertain to the altered structures of RNA from relaxed particles.

I have investigated the events leading to full methylation of immature ribosomes during ribosome development. From the evidence presented here, it may be concluded that complete methylation of rRNA occurs prior to the development of mature ribosomes. Moreover, the acquisition of the final configuration of rRNA occurs at a later step, concomitant with the final addition of protein to the developing particles.

MATERIALS AND METHODS

Bacterial cultures and media. All the cultures used here were relaxed strains of *E. coli* K-12. LMR (lysine$^-$, methionine$^-$) and LMUR (lysine$^-$, methionine$^-$ uracil$^-$) were derived by nitrosoguanidine mutagenesis from the ribonuclease-negative strain of Gesteland (7). MUR (methionine$^-$ uracil$^-$) was obtained from G. Stent, and was referred to as U-49 in his collection.

All experiments were performed in a minimal-salts medium (16), properly supplemented with required nutrients. Glucose was employed as sole carbon source.

Cell extracts and biochemical methods. The preparation of extracts in the French pressure cell, extraction of nucleic acids, analyses performed by sucrose gradients and methylated albumin-kesielguhr (MAK) chromatography, and radioactivity determinations have been described (16). RNA and protein were estimated by the methods of Dische (3) and Lowry et al. (9), respectively. Methionine-methyl-^{3}H, *S*-adenosylmethionine-methyl-^{3}H, uniformly labeled ^{14}C-uracil and uracil-*3,4*-^{3}H were obtained from Schwarz Bio Research, Inc., Orangeburg, N. Y.

In vitro methylation of RNA. The methyl-accepting capacity of RNA preparations was determined by the method of Gordon and Boman (8). The total reaction volumn was 1.0 ml. Levels of RNA were from 0.29 to 0.6 mg in the reaction. The methyl donor was *S*-adenosylmethionine-methyl-^{14}C with a specific ac-

tivity of 130 mc/mg. In each reaction mixture, 0.1 mc was used.

Chromatography of bases. RNA was hydrolyzed to bases by heating in 12 N perchloric acid at 100 C for 90 min. The preparations were cooled and neutralized with KOH. Potassium perchlorate was removed by centrifugation, and the remaining solution of hydrolysis products was dried in vacuo. The bases were separated by two-dimensional chromatography on thin-layer cellulose plates (Eastman Chromogram). The solvent for the first dimension was isopropyl alcohol-12 N HCl-water (68:16:20). The second dimension solvent was *n*-butylalcohol-formic acid-water (77:10:13). Carrier 5-methyl uracil (thymine) was mixed with hydrolysis products. Uracil, cytosine, and thymine were located and scraped from the plates. After elution with 1 N HCl from the cellulose, each compound was rechromatographed as above. Radioactivity was determined on the material eluted from the second chromatogram.

RESULTS

Methylation of RNA during amino acid deprivation. It is known that the soluble RNA accumulated during methionine deprivation of a relaxed mutant is nonmethylated (6). Since methionine serves as the methyl donor in the methylation reaction, it might be expected that the same situation is true with respect to rRNA. However, no direct measurements of the extent of methylation have been reported for rRNA formed during methionine deprivation. Such measurements were made in the present study in the following way. The relaxed mutant LMR was labeled with uracil-*2*-^{14}C, either during logarithmic growth or during methionine starvation. The RNA was recovered by phenol-detergent extraction, and rRNA was separated from the total by chromatography on MAK. The rRNA was then subjected to hydrolysis by 12 N perchloric acid to yield free bases. Bases were then separated by two-dimensional chromatography. Uracil, cytosine, and 5-methyl uracil (thymine) were eluted from the chromatogram, and the radioactivity in each compound was determined. Table 1 shows the distribution of radioactivity in these bases during growth and during methionine starvation. No significant radioactivity was found in 5-methyl-uracil from RNA labeled during starvation. Considering the level of detectability, the extent of methylation of uracil during methionine deprivation must be less than 2% of the normal level.

The methylation of RNA was measured when relaxed mutants were deprived of amino acids other than methionine. This was done by labeling with uracil-*2*-^{14}C (0.01 μc/ml and 8 μg/ml) and methionine-methyl-^{3}H (1 μc/ml and 2 μg/ml) during amino acid deprivation. For reference, similar measurements were made on a relaxed mutant during balanced growth Separate studies showed that the turnover of either label is less than 4% per hour. This was determined by continuing the amino acid deprivation during a "chase" of nonradioactive uracil and methionine. Table 2 shows the incorporation of methyl groups into RNA from methionine-methyl-^{3}H during deprivation of amino acids other than methionine. The ribosomal RNA accumulated during starvation for arginine, lysine, or histidine is methylated to only 50 to 60% of the level of normal, logarithmically growing cells. This reduction in methylation cannot be explained by dilution of the radioactive methionine by endogenous material, since the culture deprived of lysine is also auxotrophic for methionine (*see* Materials and Methods). Therefore, even though methionine is present during the amino acid

TABLE 1. *Distribution of ^{14}C-uracil incorporated into ribosomal RNA during log growth or methionine starvation*[a]

Pyrimidine	CPM eluted[b]	
	Log cells	Starved cells
Uracil	24,085	21,270
5-Methyl uracil	244	5
Cytosine	21,200	24,650

[a] Uracil-*2*-^{14}C was incorporated into the nucleic acids of MUR during logarithmic growth ("log cells") or methionine starvation ("starved cells"). The ribosomal RNA particles were isolated by MAK chromatography of phenol-detergent extracted RNA. The RNA was hydrolyzed with 12 N perchloric acid, and the bases were separated by two-dimensional chromatography.

[b] Corrected for background radioactivity.

TABLE 2. *Level of methylation of ribosomal RNA*

Condition[a]	Methyl-^{3}H/^{14}C-RNA	Per cent log[b]
Log growth	0.850	100
Methionine-starved	—	0
Arginine-starved	0.495	58
Lysine-starved	0.468	55
Histidine-starved	0.475	56

[a] Cells were labeled with ^{14}C-uracil (0.01 μc/ml and 8 μg/ml) and methionine-methyl-^{3}H (1.0 μc/ml and 2 μg/ml) except in the case of methionine deprivation. RNA was extracted by phenol-detergent from French press extracts of whole cells. Ribosomal RNA was separated by chromatography on MAK.

[b] The methyl-^{3}H/^{14}C-RNA ratio obtained under each deprivation condition is expressed as the percentage of this ratio for cells growing logarithmically.

starvation period, the RNA which accumulates in immature ribosomes is not fully methylated. As will be shown later, this RNA does become fully methylated when the immature particles become converted to mature ribosomes. Half-methylated RNA was also isolated from immature ribosomes formed during exponential growth of a stringent culture which had been subjected to isotopes for 0.1 to 0.2 of a generation time (Sypherd, *unpublished data*).

Manor and Haselkorn (10) investigated the methyl-accepting activity of RNA isolated from relaxed particles produced during histidine starvation. They concluded that this RNA was fully methylated, since is was not an efficient methyl acceptor in vitro. Since Table 2 indicates that RNA from all relaxed particles is under-methylated, the in vitro methyl-accepting activity was measured. Relaxed particles were prepared from lysine-, histidine-, and methionine-starved cells, and the RNA was isolated from each preparation. Figure 1 shows that RNA from methionine-deprived cells [RNA (met^-)] is an excellent methyl acceptor. In agreement with Manor and Haselkorn (10), the RNA isolated from relaxed particles of lysine- and histidine-starved cells accepts methyl groups at about 1% the level of RNA (met^-). These results indicate that, although RNA (lys^-) and RNA (his^-) are submethylated in vivo, they cannot be fully methylated in vitro. The reason for this is not readily apparent, but one possibility may be the need for a specific RNA conformation which is not attained in vitro.

Relaxed particles from methionine- or lysine-starved cells. The difference in the degree to which the RNA of relaxed particles may be methylated suggested that the particles themselves may be different. This could be the case, for example, if nonmethylated and half-methylated particles are progressive stages in ribosome development. To examine possible size differences, particles were accumulated during starvation for methionine or lysine. The methionine-negative, lysine-negative mutant LMR was used in this experiment. RNA was labeled with ^3H-uracil in the lysine-deprived culture and with ^{14}C-uracil in the culture deprived of methionine. When the starvation was terminated after 90 min, both cultures were mixed, and an extract was prepared. Figure 2 shows that there are no obvious differences in the particle classes formed under these two conditions. Furthermore, MAK profiles of RNA from these particles are indistinguishable from each other and are characteristic of those reported by Sypherd and Fansler (16) for immature ribosomal RNA (Fig. 3). Whatever the effect of partial methylation, it is too subtle to be reflected in these physical properties of either the ribonucleoprotein particles or the RNA.

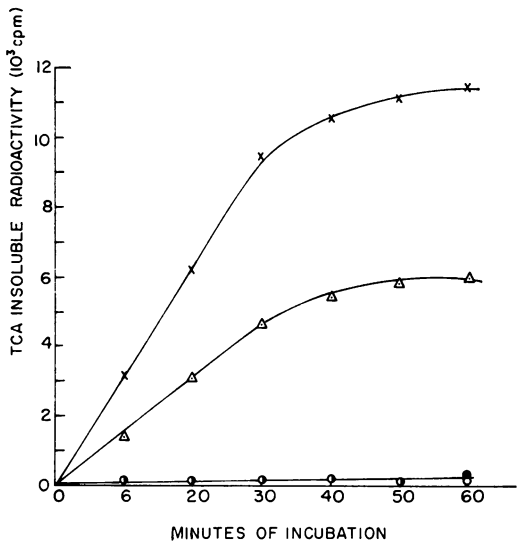

FIG. 1. *In vitro methylation of relaxed particle RNA. The methyl donor is S-adenosyl-methionine-^{14}C (see Materials and Methods). RNA was isolated from the particles of methionine-starved cells (×, 0.58 mg of RNA; △, 0.29 mg of RNA), lysine-starved cells (○, 0.6 mg of RNA), and histidine-starved cells (●, 0.55 mg of RNA). Samples (0.2 ml) were taken at intervals and treated with 5% trichloroacetic acid, and the radioactivity in the precipitate was determined.*

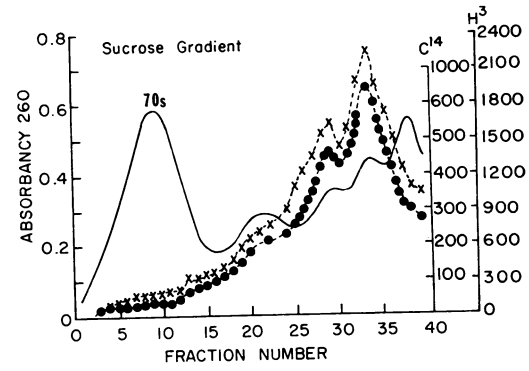

FIG. 2. *Sucrose gradient analysis of crude extracts prepared from methionine- or lysine-starved cells. The extracts were mixed and analyzed in a single sucrose gradient. On the left ordinate is plotted absorbancy at 260 nanometers (solid line). Symbols: ×, radioactivity from ^3H-uracil in RNA of lysine-starved cells; ●, radioactivity from ^{14}C-uracil in RNA of methionine-starved cells.*

Kinetics of events in relaxed-particle conversion to ribosomes. There are several events which

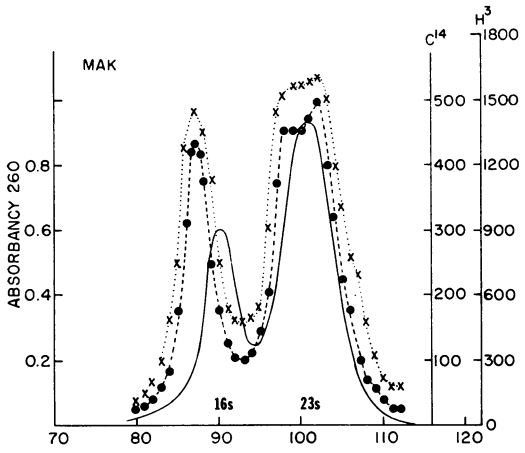

FIG. 3. *Chromatography of RNA on MAK. The RNA was isolated from the extracts shown in Fig. 2. Only the 16 and 23S RNA species are shown. The solid line is absorbancy at 260 manometers. Symbols:* ×, *radioactivity from* ^{3}*H-uracil in RNA of lysine-starved cells;* ●, *radioactivity from* ^{14}*C-uracil of RNA from methionine-starved cells.*

occur during the formation of mature ribosomes from relaxed particles. Among these are (i) *conversion* of immature particles to ribosomes by the addition of ribosomal proteins, (ii) *transition* in the structure of ribosomal RNA to the configuration found in mature ribosomes, and (iii) complete *methylation* of ribosomal RNA. In an effort to understand how these processes are related temporally, the kinetics of each was measured during the conversion of relaxed particles to ribosomes after the amino acid starvation had been relieved. In these experiments, it was important to identify only those events concerned with the accumulated relaxed particles. It has been shown that relieving the amino acid starvation results not only in conversion of relaxed particles to ribosomes, but also in the formation of more precursor particles from newly formed RNA (12). Therefore, to prevent further RNA synthesis upon restoration of the amino acid, uracil was withheld from a uracil-methionine double auxotroph. Relaxed particles were accumulated during methionine deprivation in the presence of ^{14}C-uracil. After 90 min of methionine starvation, uracil was withdrawn and methionine-(methyl-^{3}H) was added to the culture. Radioactivity from ^{14}C-uracil represents the accumulated particles, and radioactivity from methionine-(methyl-^{3}H) serves as a measurement of the methylation of RNA in these particles during the maturation process. During the methionine$^+$, uracil$^-$ stage, samples were removed and subjected to the following analyses: (i) sucrose-gradient sedimentation was used as a measure of the rate of particle *conversion* to ribosomes; (ii) RNA isolated by phenol-detergent extraction was chromatographed on MAK columns, to determine the rate of methylation of ribosomal RNA; and (iii) MAK column analyses also yielded the rate at which relaxed-particle RNA underwent transition to mature rRNA. This was demonstrated by determining the amount of radioactivity from relaxed-particle RNA which did not chromatograph within the 16*S* peak of mature rRNA. This value was then normalized as the percentage of noncoincident 16*S* RNA.

The data derived from both sucrose-gradient and MAK analyses are represented in kinetic plots in Fig. 4 and 5. In an effort to expand the time scale in the kinetic study, the three processes were measured at 22 C on relaxed particles which had been formed at 37 C. Figure 5 shows that the kinetics for methylation, conversion, and transition are qualitatively the same as those in Fig. 4. Figures 4 and 5 show that relaxed particles are converted to ribosomes after a lag, whereas polymer methylation occurs linearly from the time methionine is added to the culture. The rate at which the transition in RNA structure occurs falls on the line describing the rate of conversion of relaxed particles to ribosomes.

The increase in the number of methyl groups in RNA is a function of T^1, which is consistent with

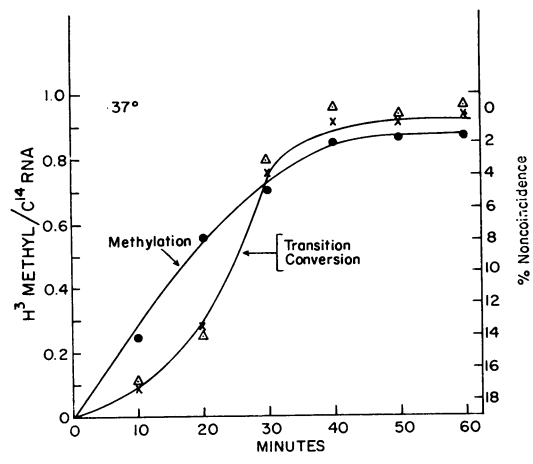

FIG. 4. *Kinetics of methylation, particle conversion, and transition in RNA structure. Particles were accumulated at 37 C during methionine deprivation in the presence of* ^{14}*C-uracil (0.01 μc/ml and 4 μg/ml) in MUR. Uracil was then removed from the medium, and methionine-methyl-*^{3}*H (1 μc/ml and 2 μg/ml) was added to terminate the amino acid starvation. The cells were placed at 37 C, and samples were removed and extracted to measure the three parameters.*

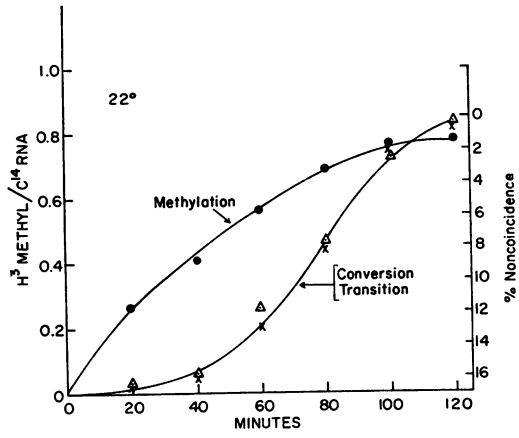

FIG. 5. *Kinetics of methylation, particle conversion, and transition in RNA structure. Particles were accumulated at 37 C as in Fig. 4. After the removal of uracil and addition of methionine-methyl-³H, the cells were placed at 22 C.*

a single step in the methylation of relaxed-particle RNA. However, both conversion of particles and transition in RNA structure occur as functions of T^2, suggesting that at least one other step precedes these two events (2). These data imply that methylation precedes particle conversion, and does not occur concomitantly with conversion or transition. Since methylation of RNA and the transition in RNA structure are described by different time constants, it may be concluded that they are not causally related.

Formation of fully methylated relaxed particles. In the preceding sections, it was shown that relaxed particles can exist in at least two forms with respect to methylation: nonmethylated in methionine-starved cells and half-methylated in cells deprived of amino acids other than methionine. The kinetic experiments shown in Fig. 4 and 5 suggest that, to achieve complete methylation of the precursor particles, there is a requirement for either protein synthesis or for the presence of all amino acids. The following experiments represent a successful attempt to achieve complete methylation of relaxed particles.

Submethylated relaxed particles were accumulated in the absence of lysine in a relaxed mutant auxotrophic for lysine, methionine, and uracil. ¹⁴C-uracil and methionine-methyl-³H were present to label relaxed RNA and methyl groups to the 50% level, respectively. Further methylation of these relaxed particles was then studied at 22 C in the absence of uracil under the following conditions: (i) during and after a 20-min supply of lysine; (ii) during a 100-min incubation with lysine and 200 μg of chloramphenicol per ml; and (iii) during a 100-min incubation in the absence of lysine and uracil.

Methionine-methyl-³H was present in all cases. No degradation of RNA was detectable under these conditions. The results of these experiments are shown in Fig. 6, where it can be seen that full methylation of relaxed particle RNA occurred in those cells which had been provided lysine for 20 min. No conversion or transition occurred during the incubation period (Fig. 7 and 8). Incubation with lysine and chloramphenicol severely inhibited further methylation. Similarly, continued incubation in the absence of lysine and uracil resulted in little further methylation of the RNA. From these experiments, the following conclusions were made: (i) relaxed particles can be fully methylated after a period of protein synthesis, but before there is significant conversion of particles to mature ribosomes (Fig. 6, culture a; Fig. 7); (ii) the peculiar chromatographic characteristics of relaxed-particle RNA are independent of the level of methylation (Fig. 8); (iii) the presence of the essential amino acid is itself not sufficient to permit complete methylation of the relaxed-particle RNA, since chloramphenicol pre-

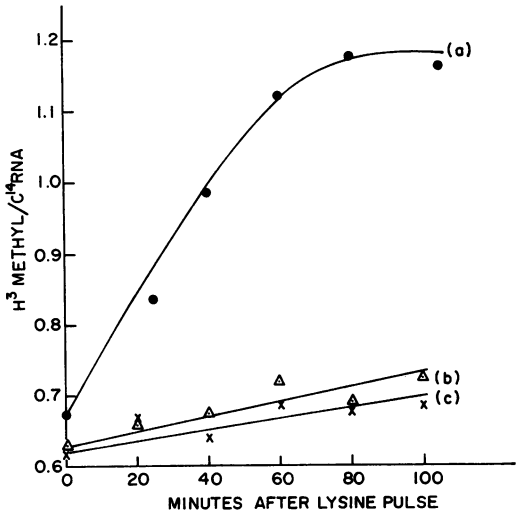

FIG. 6. *In vivo methylation of submethylated particles. Strain LMUR was starved for lysine at 37 C in the presence of ¹⁴C-uracil and methionine-methyl-³H. After 60 min of starvation, uracil was removed, the culture was divided into three parts, and incubation was continued at 22 C. Each portion was treated as follows. Culture (a) was incubated with a 20-min supply of lysine (lysine pulse) and continued for 100 min after the lysine was exhausted. Culture (b) was incubated with excess lysine and 200 μg of chloramphenicol per ml for 120 min. Culture (c) was incubated without lysine and without uracil for 120 min. Excess methionine-methyl-³H was present in all three cultures.*

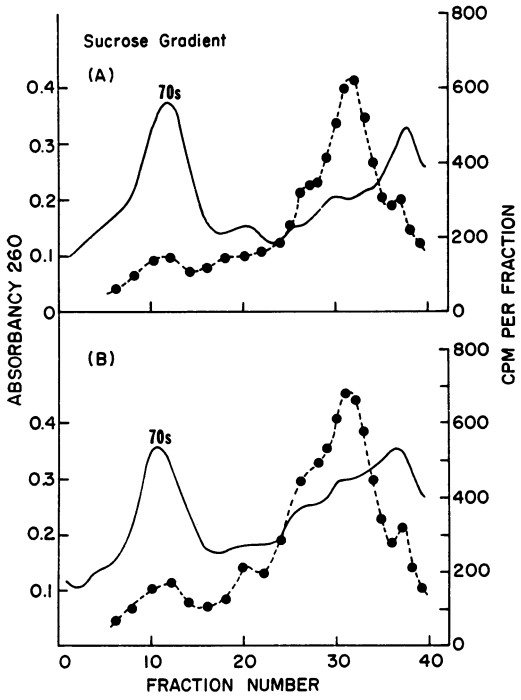

FIG. 7. *Sucrose gradient analyses of crude extracts prepared from culture (a) in Fig. 6 before (A) and 100 min after (B) a 20-min lysine pulse. Solid line, absorbancy at 260 nanometers; broken line, radioactivity from* ^{14}C*-uracil.*

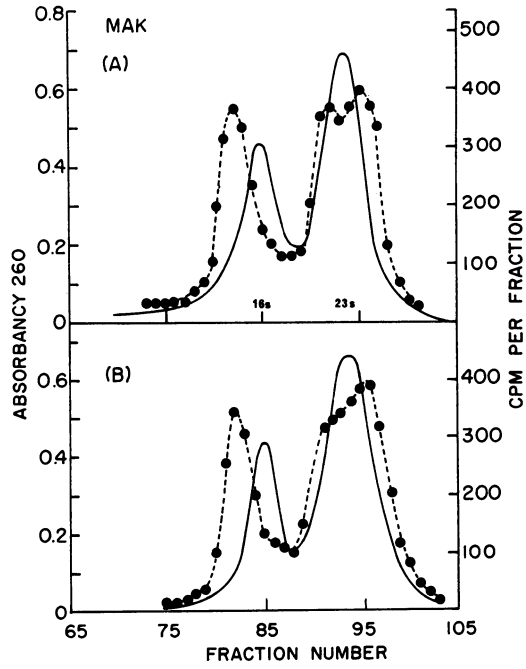

FIG. 8. *MAK analyses of RNA extracted from culture (a) in Fig. 6 before (A) and 100 min after (B) a 20-min lysine pulse. Solid line, absorbancy at 260 manometers; broken line, radioactivity from* ^{14}C*-uracil.*

vents further methylation (Fig. 6, culture b); if further RNA synthesis is halted during relaxed starvation for lysine, there is no increase in the level of methylation of relaxed-particle RNA (Fig. 6, culture c).

DISCUSSION

The process of ribosome biosynthesis involves the integration of nucleic acid and several proteins into a complex particle. The overall process of integration includes a complicated series of reactions involving the synthesis of both macromolecular species. It is known that the ribosomal RNA molecules (16*S* and 23*S*) are synthesized as intact, continuous polynucleotides (17), with methylation of certain bases occurring after polymerization of the nucleotides (6, 8). A transition in structure, or perhaps more accurately the acquisition of final configuration (15), may be the terminal event in the series of reactions leading to RNA integration into mature ribosomes.

The purpose of the present study was to relate the methylation of ribosomal RNA to the structural transition which occurs in the RNA during ribosome development. The experiments reported here show that methylation may occur in two stages. One stage is early in the development of the ribonucleoprotein particle, and leads to the formation of half-methylated RNA. This methylation step is not obligatory, since it can be bypassed during methionine deprivation. The second stage of methylation is just prior to the final maturation process, which occurs by the addition of protein to the immature particle. The results of the kinetic study, in which the complete methylation and the structural transition are described by different rate constants, suggest that there is no causal relationship between methylation and transition. This suggestion is supported by the accumulation of fully methylated relaxed particles which contain immature RNA. It is concluded that submethylation of the polymer does not directly account for the structural properties (15) of relaxed-particle RNA.

The integration of proteins into the developing ribosome occurs in several steps. This is known from the kinetic studies of Roberts and his collaborators (1), and from the protein deficiency of relaxed particles (13). The ribosomal proteins are synthesized on pre-existing ribosomes and are transferred to the developing particle (14) in apparently discrete steps. The mechanism for

ordering the proteins in the proper sequence is unknown, but may point out the functional significance of the heterogeneity of ribosomal proteins.

Unpublished experiments from this laboratory show that relaxed particles are deficient in the most basic protein components. Presumably, these are the last proteins to become incorporated into the particle. These few proteins seem to confer two properties upon the ribosomal RNA after their integration into the particle. They render the RNA capable of becoming fully methylated (hence, the requirement for some protein synthesis as shown in Fig. 6), and they allow the RNA to achieve its final structural configuration. These proteins could direct the tertiary structure of the RNA by restricting configurations which could otherwise be attained by random folding of the polynucleotide chain. However, proof for such a role of ribosomal proteins may depend upon successful reconstitution studies in which the constituent rRNA and proteins can be assembled in vitro.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant GM 12686 from the National Institute of General Medical Sciences and by National Science Foundation grant GB 5901.

I gratefully acknowledge the capable and cheerful assistance of Lida Bilokur.

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