Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 7144–7148, October 1988
Biochemistry

Coregulation of processing and translation: Mature 5′ termini of *Escherichia coli* 23S ribosomal RNA form in polysomes

(rRNA processing/ribosomes/maturation/protein synthesis)

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Communicated by Norton D. Zinder, June 30, 1988

ABSTRACT **In *Escherichia coli*, the final maturation of rRNA occurs in precursor particles, and recent experiments have suggested that ongoing protein synthesis may somehow be required for maturation to occur. The protein synthesis requirement for the formation of the 5′ terminus of 23S rRNA has been clarified *in vitro* by varying the substrate of the reaction. In cell extracts, pre-23S rRNA in free ribosomes was not matured, but that in polysomes was efficiently processed. The reaction occurred in polysomes without the need for an energy source or other additives required for protein synthesis. Furthermore, when polysomes were dissociated into ribosomal subunits, they were no longer substrates for maturation; but the ribosomes became substrates again when they once more were incubated in the conditions for protein synthesis. All of these results are consistent with the notion that protein synthesis serves to form a polysomal complex that is the true substrate for maturation. Ribosomes in polysomes, possibly in the form of 70S initiation complexes, may more easily adopt a conformation that facilitates maturation cleavage. As a result, the rates of ribosome formation and protein synthesis could be coregulated.**

Since cleavage and trimming of large precursors to mature ribosomal RNAs naturally precedes function of the rRNA in ribosomes (1), it has been generally thought that the production and activity of ribosomes are distinct and noninteractive processes. However, recent investigations in *Escherichia coli* suggest a connection between the synthesis of proteins and the maturation of 16S and 23S rRNA (2, 3). In a sense, it is obvious that processing and function of ribosomes must be reciprocally dependent. Since many precursors, particularly those of 16S rRNA, cannot form active ribosomes (4, 5), processing is required for the continued expansion of protein synthetic capacity in cells. Also, the final reactions in rRNA processing must take place on preribosomes, which are complexes of pre-rRNA and ribosomal proteins (6); and processing is therefore dependent on the continued production of ribosomal protein.

It is not surprising, then, that the processing of rRNA chains stops short of completion when protein synthesis is blocked by antibiotics like chloramphenicol (7). Fig. 1*A* shows a schematic of processing of wild-type *E. coli* 23S rRNA. In the presence of chloramphenicol, processing stops after RNase III has cleaved the double-stranded stem of the large precursor. A similar process is observed in 16S rRNA maturation, again with the formation of a truncated double-stranded stem-and-loop structure (1).

The inhibition of rRNA maturation when protein synthesis is blocked has shown, however, several unexpected features. First, the inhibition of maturation is essentially immediate *in vivo*, even though cells contain appreciable pools of already-assembled preribosomes [up to 10% of the total ribosome population (1)] and free pools of individual ribosomal proteins (12). Second, several investigations have shown that the formation of certain mature termini, including the 5′ ends of both 16S and 23S rRNA, proceeds more efficiently under protein synthetic conditions *in vitro*. Possibly one of the components of protein synthetic mixtures, like GTP, might be required for the maturation reaction; or preribosomes, which have been found often in polysomes (13–16), might begin to participate in some partial reactions of protein synthesis and thereby achieve an RNA conformation required for the maturation reaction.

To try to clarify the interrelationship of protein synthesis and rRNA maturation, we have studied an RNase III-deficient strain. In that mutant, 23S rRNA processing fails, but the precursor rRNAs are active enough to support protein synthesis (9). Some cleavages occur in the primary transcript in the mutant, but they result in rRNA chains predominantly much longer than the wild-type chains (with 5′ termini at A, C1, and C2 in Fig. 1*A*, as in ref. 9). In contrast, the 5′ terminus of 23S pre-rRNA in mutant 50S or 70S ribosomes was processed to some extent by protein preparations from wild-type cells in protein synthetic conditions (3). We have now shown that the maturation reaction can be carried out efficiently in the absence of protein synthetic conditions if polysomes rather than ribosomes are the substrate. This suggests that the final maturation step occurs only after preribosomes join in polysomes, linking the formation of ribosomes directly to their incorporation into the protein synthetic machinery.

MATERIAL AND METHODS

Bacterial Strains and Growth Conditions. *E. coli* strain D10 (17) was grown at 37°C in Luria broth to an optical density of 0.55–0.65 at 550 nm. The cells were harvested on ice, washed with ice-cold buffer (10 mM Tris·HCl, pH 7.4/5 mM $MgCl_2$/2 mM $CaCl_2$), and stored at −70°C until use. Strain ABL1, deficient in RNase III (18), was grown in broth at 30°C.

Preparation of the Ribosome Wash. To prepare the ribosome wash, a crude extract (9) from 2.5 g of *E. coli* D10 cells in buffer A (20 mM Tris·HCl, pH 7.8/60 mM NH_4Cl/10 mM $Mg(OAc)_2$/10 mM 2-mercaptoethanol) was centrifuged over a layer of 30% sucrose in buffer A at 105,000 × *g* for 3 hr in a Beckman type 65 fixed-angle rotor. The upper two-thirds of the supernatant was collected (as the S100 fraction; the source of soluble factors), dialyzed against 10 mM Tris·HCl, pH 7.6/10 mM $Mg(OAc)_2$/6 mM 2-mercaptoethanol, centrifuged at 10,000 × *g* for 10 min to remove debris, and then stored frozen at −20°C. The ribosome pellet was resuspended in buffer A, but with 1 M NH_4Cl, for 1 hr at 0°C. Ultracentrifugation was repeated, and the upper 85% of the supernatant phase was recovered. Proteins were precipitated

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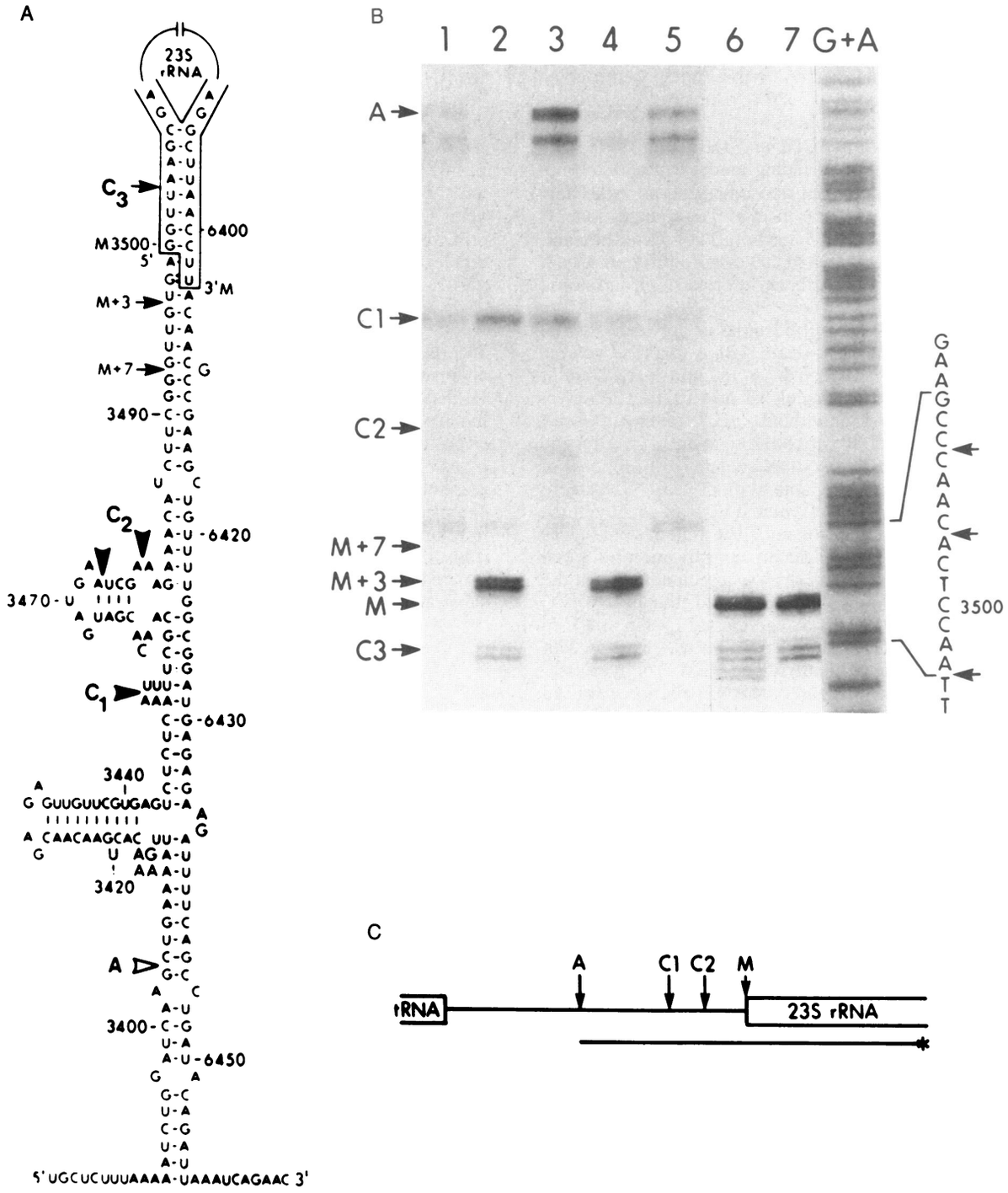

FIG. 1. (*A*) Secondary structure of pre-rRNA sequences adjacent to the 5′ (nucleotide 3500) and 3′ (nucleotide 6403) termini of 23S rRNA. The open arrowhead (A) indicates the amount of sequence included in the *rrnB* operon probe used for S1 nuclease protection experiments in *B*. RNase III cleavages proximal to 23S rRNA at M + 3 and M + 7 (three and seven nucleotides longer than the mature 5′ end, M), which dominate in wild-type cells (3), and at C3 (8) are indicated by closed arrows. Closed arrowheads C1 and C2 indicate cleavage sites that predominate in the RNase III-deficient strain (9). Nucleotide numbers are as in ref. 10. (*B*) Maturation of the 5′ end of 23S rRNA *in vitro*: S1 nuclease protection assay. Lanes 1, 3, and 5 show the termini observed in 23S rRNA of the RNase III-deficient strain ABL1; the RNA was incubated in buffer either as isolated 23S pre-rRNA (lane 1) or in 50S ribosomal particles (lane 3) or polysomes (lane 5). Lanes 2, 4, and 6 show the termini after reactions of each of the samples in lanes 1, 3, and 5 for 120 min at 37°C with the ribosome wash from a wild-type strain (D10). Total RNA from the reaction mixtures was extracted, and the S1 nuclease protection assay was carried out. Lane 7 shows the termini observed in the control 23S rRNA from wild-type strain D10. A sequencing lane (G + A) verifies the location of the cleavages along the known sequence of *rrnB*; here closed arrows indicate the known sites of cleavage by RNase III (ref. 3). (*C*) Hybridization probe for S1 nuclease mapping of 23S pre-rRNA. The probe spans the mature 5′ terminus (nucleotides 3404–3606 in ref. 10) and is end-labeled at the end within mature rRNA (11).

with saturated ammonium sulfate (0.4 g/ml), and the pellet was dissolved in 1 ml of 20 mM Tris·HCl, pH 7.6/50 mM NH_4Cl/5 mM 2-mercaptoethanol/0.1 mM EDTA/10% (vol/vol) glycerol. After centrifugation to remove debris, the clear solution was stored frozen at −20°C.

Preparation of Polysomes, Ribosomes, and rRNA. Polysomes were prepared from strain ABL1 (RNase III-deficient) as described (8) except that the crude lysate was centrifuged through the gradient of 10–30% sucrose for a longer time (35,000 × *g* for 15 hr). In a replicate gradient, 30S, 50S, and

70S ribosomes were centrifuged as markers. Monosomes and polysomes of different size were localized in gradient fractions by measuring absorbance at 260 nm and monitoring radioactivity. Fractions containing polysomes were pooled and stored in 50% glycerol at −20°C. The largest polysomes were used for maturation studies.

Ribosomes from strain ABL1 and D10 (wild-type) were also prepared by alumina grinding as described (9). Ribosomal RNA was isolated from ribosomes and polysomes by phenol extraction in the presence of 1% sodium dodecyl sulfate. The RNA was precipitated with 2 vol of cold ethanol, pelleted by centrifugation at 10,000 × *g* for 15 min, dried, dissolved in hybridization buffer as described (11), and stored at −20°C.

Maturation Assay *in Vitro*. Ribosomes or polysomes (1 μg) from the RNase III-deficient strain ABL1, in a total volume of 50 μl of 10 mM Tris·HCl, pH 7.6/180 mM NH_4Cl/8 mM $MgCl_2$/5 mM 2-mercaptoethanol/0.1 mM EDTA/10% glycerol were incubated with or without 5 μl of "ribosome wash" from wild-type strain D10 for 60–120 min at 37°C. After the reaction, 1/3rd vol of 1 M sodium acetate (pH 4.6), 5 μl of 10% sodium dodecyl sulfate, and 10 μg of yeast tRNA were added, and the mixture was extracted with an equal volume of phenol/chloroform, 1:1 (vol/vol). The aqueous phase was separated from the phenol layer by centrifugation for 3 min in an Eppendorf centrifuge. RNA was precipitated from the aqueous phase by addition of 2.5 vol of cold ethanol at −45°C for at least 30 min. After centrifugation for 15 min at 4°C in an Eppendorf centrifuge, the RNA pellet was washed with cold 70% ethanol, dried, and dissolved in 20 μl of hybridization buffer. Five to ten microliters of RNA solution was used for each assay; the remainder was stored at −20°C until used.

S1 Nuclease Protection Assay. Hybridization of the end-labeled single-stranded DNA fragment (3000–5000 cpm) complementary to the 5′ terminus of 23S pre-RNA (see ref. 11 and the legend to Fig. 1) and subsequent S1 nuclease treatment followed described procedures (3, 9). The S1 nuclease-resistant nucleic acids were resuspended in 10 μl of 90% deionized formamide containing 0.5% each of the size markers xylene cyanol and bromophenol blue, and fractionation of the products was carried out in a 45 cm long × 0.5 mm thick 8% polyacrylamide (30:1 acrylamide/methylenebisacrylamide) gel containing 8.3 M urea and 90 mM Tris borate (pH 8.0).

Primer Extension Assay. rRNA (0.2–0.4 μg) was hybridized to the primer (8,000–10,000 cpm; see the legend to Fig. 3) in 20 μl of hybridization buffer under the conditions described for S1 nuclease protection assays. After 3 hr at 65°C, the hybridized nucleic acids were precipitated and redissolved in reverse transcriptase buffer, and the primer was extended on the RNA template with 14 units of avian myeloblastosis virus reverse transcriptase for 1 hr at 41°C as described (19). The solution was brought to 0.025 M EDTA and 0.2 M NaOH and incubated for an additional 30 min at 37°C. Twelve microliters of 1 M Tris·HCl (pH 7.5) and 5 μg of yeast tRNA were added, and the volume was brought to 200 μl with water. The nucleic acids were then extracted with phenol and precipitated with ethanol. The products were analyzed in an 8% polyacrylamide/urea gel as described above.

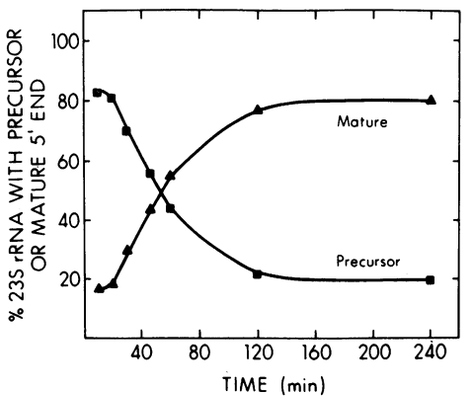

FIG. 2. Time course of the conversion of the precursor to the mature 5′ termini of 23S rRNA. The relative amounts of precursor (M+3) and mature (M) termini in polysomal RNA are plotted as a function of the time of incubation with the ribosome wash as in Fig. 1. Quantitation of each species was determined by densitometric tracings of an autoradiogram like that in Fig. 1*B*; specific signals were measured as a fraction of the total signal observed from each lane.

RESULTS AND DISCUSSION

50S and 70S ribosomes and polysomes were obtained by sucrose gradient fractionation from extracts of the RNase III-deficient strain. Since there is no background of wild-type mature termini in these cells, the conversion of 23S pre-rRNA to mature rRNA can be detected easily in cell extracts by using 50S ribosomes from the mutant as a substrate. The reactions can be followed by using S1 nuclease (3) or primer extension (20) assays of the termini produced. The ribosome fractions were treated in buffered salt solutions with a ribosome wash preparation made from wild-type cells. When isolated 23S pre-rRNA or 50S ribosomes were used as substrates, no mature termini were produced. Only termini

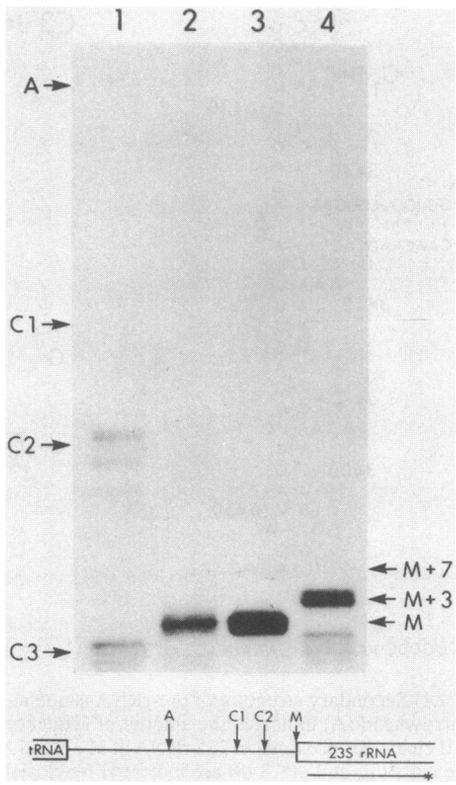

FIG. 3. Primer extension analysis (20) of 5′ termini of 23S rRNA species extracted from polysomes before (lane 1) and after (lane 2) reaction with the ribosome wash. Lane 3 shows the control 5′ termini from wild-type strain D10, and lane 4 shows the rRNA termini in 50S ribosomes after the reaction. M, M+3, and M+7 species are those produced by RNase III (ref. 3); A, C1, C2, and C3 are as in Fig. 1*A*. The single-stranded primer, complementary to mature 23S rRNA nucleotides 3606–3513, was ^{32}P-end-labeled at nucleotide 3606 (scheme at the bottom). RNA was extracted from treated or untreated ribosomes and hybridized with primer, and the extension reaction was carried out with avian myeloblastosis virus reverse transcriptase. The products were fractionated on an 8% denaturing gel as in Fig. 1*B*.

Table 1. Maturation of the 5′ terminus of 23S rRNA in polysomes

Substrate	23S rRNA 5′ termini, %		
	M+7	M+3	M
Polysomes from strain ABL1			
Untreated	—	—	—
Treated in buffer	—	10	90
Treated in buffer after high [Mg^{2+}] dialysis	—	15	85
Treated in buffer after low [Mg^{2+}] dialysis	5	95	—
Treated in buffer after low [Mg^{2+}] dialysis and subsequent preincubation in high [Mg^{2+}]	5	95	—
Dialyzed in low [Mg^{2+}] and treated after incubation under protein synthesis conditions	5	55	40
Control RNA from wild-type strain D10	7	2	91
70S ribosomes treated in buffer*	10	90	—

Polysomes were dialyzed against 20 mM Tris·HCl, pH 7.6/160 mM NH_4Cl/5 mM 2-mercaptoethanol/10% glycerol containing 1 mM ("low") or 8 mM ("high") $MgCl_2$ as indicated. Ribosomes were incubated under conditions of protein synthesis as in ref. 22. Subsequent treatment in buffer, RNA extractions, and S1 nuclease protection analysis were as in Fig. 1*B*.
*Cells were ground with alumina, and 70S ribosomes were isolated from the crude extract by zonal sedimentation in sucrose gradients.

that were seven and three nucleotides longer than the mature 5′ end, M, were produced (Fig. 1*B*, lanes 2 and 4 compared with lanes 1 and 3 for the untreated samples). These results are characteristic of RNase III action (19). Similar termini were produced by incubating purified RNase III with purified 23S pre-rRNA (3) or with 50S or 70S ribosomes (see ref. 3 and Table 1, line 8).

In contrast to the results with isolated 23S pre-rRNA and ribosomes, mature termini were efficiently formed when polysomes were incubated with the ribosome wash (Fig. 1*B*, lane 6; compare the termini in untreated polysomes in lane 5). The pattern of fragments observed is complex, including the predominant 5′ mature end (and other termini like "C3"; see ref. 8); the important point, however, is that the species produced are indistinguishable from those formed during the maturation of wild-type rRNA (Fig. 1*B*, lane 7).

Fig. 2 shows the kinetics of processing of pre-rRNA to the mature species by the ribosome wash fraction. The conversion is slow but is essentially complete after about 100 min. Detailed kinetics show that the M+3 terminus is formed rapidly by RNase III, but the final cleavage to form the mature end is much slower. The rate is comparable in polysomes incubated in buffer or in complete protein synthetic conditions and is strongly dependent on divalent (Mg^{2+}) and monovalent (NH_4^+ or K^+) ions [optimal concentrations are 8 mM and 180 mM, respectively (data not shown)].

Fig. 3 shows an independent confirmation of these findings in a primer extension assay. The long precursors seen in untreated polysomes (Fig. 3, lane 1) are processed to prominent products (Fig. 3, lane 2) with the mature terminus seen in wild-type ribosomes (Fig. 3, lane 3). The incomplete maturation of 50S ribosomal RNA in similar conditions is seen in lane 4; the initial reactions with RNase III have gone essentially to completion, yielding traces of the immature M+7, much of the M+3, and some of the M−4 ("C3") species, but no mature rRNA.

To test further the notion that polysomes and not simply ribosomes are the favored substrate for the maturation step, polysomes were dialyzed against a concentration of Mg^{2+} low enough to dissociate them to ribosomal subunits (but not low enough to inactivate the ribosomes for 70S ribosome formation; ref. 21). Table 1 (lines 4 and 5) shows that the ribosomes obtained were no longer substrates for the maturation reaction. Dialysis itself has no damaging effect on the ribosomes, since ribosomes dialyzed against high Mg^{2+} levels remained an active substrate (Table 1, line 3). Consistent with the notion that protein synthesis is required to reform polysomes, ribosomes that were dialyzed (and thereby failed as substrates for maturation) again became substrates when they were incubated under protein synthetic conditions (Table 1, line 6 compared with line 2). The recovery of activity was incomplete in the three experiments performed, perhaps because efficient mobilization of free ribosomes into polysomes in subcellular systems rarely exceeds 30% (ref. 23).

The simplest interpretation of these results is that ribosomes adopt a conformation appropriate for the maturation reaction when they are in polysomes and lose that conformation when they are in the form of 50S ribosomes. At present there is no evidence that ribosomes can form at all from isolated preribosomes, and we infer that instead a polysomal intermediate seems to be involved (Fig. 4).

The mature 5′ terminus of 23S rRNA does not form when polysomes are incubated without the ribosome wash (Table 1) or when they are incubated with purified RNase III alone (data not shown). Thus, the reaction is not a further activity of RNase III, nor is it autocatalytic in an obvious way. A soluble enzyme or factor seems to be required.

The exact nature of the ribosomal substrate for maturation is still unclear. In a previously reported case, the RNase colicin E3 is inactive with free 30S ribosomal subunits but cleaves a site in the 16S rRNA of 30S ribosomal subunits that are complexed with 50S subunits (24, 25). In a somewhat analogous way here, pre-23S rRNA is matured in 50S ribosomal subunits complexed in polysomes, but free 50S or

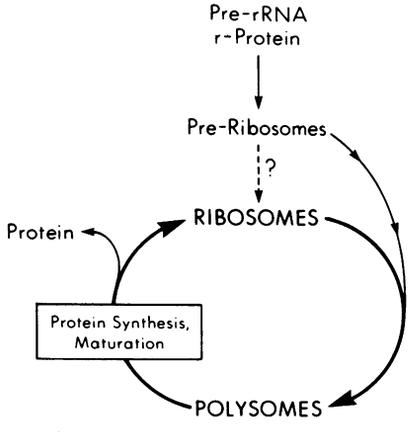

FIG. 4. Probable pathway of final maturation of rRNA in relation to protein synthesis.

70S ribosomes are not substrates (Table 1, lines 4, 5, and 8). It seems possible that the true substrate for maturation is the 70S initiation complex. The complex normally forms by the successive accretion of mRNA, initiation factors, initiator tRNA, and 50S ribosomes combined with a 30S ribosome; an analogous complex containing preribosomes may be an adequate substrate for processing reactions.

Our results suggest a basis for the presence of pre-rRNAs in polysome preparations. In addition to 16S and 23S rRNA, bacterial 5S rRNA probably matures after ribosomes are nearly completely formed (13–16). Even in eukaryotic yeast cells, in which ribosome assembly occurs in nucleoli, the final maturation of 18S rRNA has been reported to occur somewhere in the cytoplasm (26). We propose that in all of these cases preribosomes may join to mRNA before maturation is complete (Fig. 4). This implies that the rates of protein synthesis and rRNA processing may be interrelated or mutually controlled in a much more intimate way than seemed likely before. Gourse *et al.* (27) have suggested that mature ribosomes may regulate ribosome formation at the level of rRNA transcription; but bacteria might also regulate the quantity and rate of production of ribosomes at the level of maturation. The rate of protein synthesis may directly limit the rate of processing; and immature ribosomes, in turn, may even limit the movement of mature competent ribosomes on mRNA.

Such a link between processing and maturation would be important for the regulation of cell physiology if polysomes containing pre-rRNA translate less efficiently than matured polysomes. *In vivo* studies are suggestive: RNase III-deficient strains, which contain polysomes with only precursors to 23S pre-rRNA, grow more slowly and show defects in the translation of β-galactosidase and other mRNAs (28–30). These defects are corrected when RNase III is restored to the strains; but the effects could be indirect rather than based directly on altered polysome function. Definitive tests of the efficiency of immature and mature polysomes await the further purification of the required processing factors.

We thank Ravi Sirdeshmukh for his help in initiating this project, which is supported by National Science Foundation Grant PMS PCM8406949.

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