rRNA Maturation as a “Quality” Control Step in Ribosomal Subunit Assembly in *Dictyostelium discoideum*

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In *Dictyostelium discoideum*, newly assembled ribosomal subunits enter polyribosomes while they still contain immature rRNA. rRNA maturation requires the engagement of the subunits in protein synthesis and leads to stabilization of their structure. Maturation of pre-17 S rRNA occurs only after the newly formed 40 S ribosomal particle has entered an 80 S ribosome and participated at least in the formation of one peptide bond or in one translocation event; maturation of pre-26 S rRNA requires the presence on the 80 S particle of a peptidyl-tRNA containing at least 6 amino acids. Newly assembled particles that cannot fulfill these requirements for structural reasons are disassembled into free immature rRNA and ribosomal proteins.

In all organisms, rRNA molecules are transcribed from large operons (1–7), presumably because they must be synthesized in stoichiometric amounts. However, they are not excised from the multicistronic transcript in a single step, but are processed to their final size through sequential steps involving both endonucleases and exonucleases (8–19). The significance of this process is unknown.

We have recently reported that functional eukaryotic ribosomal particles can be reconstituted in vitro from free rRNA and ribosomal proteins (20), as was shown almost 30 years ago for bacterial ribosomal subunits (21, 22). In the eukaryotic system that we have explored (*Dictyostelium discoideum*), as in *Escherichia coli* (23), immature rRNA is a much better substrate for in vitro ribosome assembly. This is in agreement with the finding that in *D. discoideum*, as well as in *E. coli* (24) and yeast (25), ribosomal particles newly assembled in vivo apparently have a full complement of ribosomal proteins, but still contain immature rRNA. Furthermore, as shown for *E. coli* (26), ribosomal particles of *D. discoideum* containing immature rRNA either reconstituted in vitro or newly assembled in vivo are fully functional in protein synthesis.

These data indicate that although rRNA processing may be initiated before transcription is completed (27), rRNA maturation occurs after (and not during) ribosomal subunit assembly. We have now analyzed some structural, functional, and metabolic properties of immature ribosomal subunits, and on the basis of the data reported here, we suggest that rRNA maturation is part of a mechanism by which cells control the functionality of newly assembled ribosomal particles before locking them in an irreversible configuration. Ribosomal particles that cannot function in protein synthesis because they are not assembled correctly do not undergo maturation of the RNA and are subsequently disassembled into their components.

**EXPERIMENTAL PROCEDURES**

**Cell Culture Conditions—** *D. discoideum* AX2 cells were grown as described previously (28).

**Labeling Conditions—** For labeling with uracil, cells were usually removed from the growth medium and resuspended in Sorenson buffer at a concentration of $2 \times 10^8$/ml. For labeling with $[32P]$orthophosphate, cells were resuspended in MES-PDF buffer (29).

**Cell Fractionation—** Cell fractionation was carried out as described (20).

**Isolation of Ribosomal Subunits and Polyribosomes—** The procedure followed is described in Ref. 30.

**Gel Electrophoretic Analysis of Ribosomal RNA—** $^3H$- and $^{14}C$-labeled rRNAs were extracted from ribosomal subunits with Ultraspec II RNA following the instructions of the manufacturer (Fluka). When needed, they were mixed together in an appropriate ratio, denatured by heating at 65 °C in 50% formamide for 5 min, and analyzed by electrophoresis on a 5% polyacrylamide gel in 6 M urea. At the end of the run, the gel was cut into 1.5-mm slices, which were counted in Triton solution (Packard Instrument Co.).

**Preparation of AC914 mRNA—** The RNA was isolated by the hybrid release procedure as described (31).

**In Vitro Reconstitution of Ribosomal Subunits—** The procedure followed is described in Ref. 20.

**In Vitro Protein Synthesis System—** This was as described in Ref. 20.

**RESULTS**

**Immature Ribosomal Subunits Enter Polyribosomes with the Same Efficiency as Mature Particles—** In previous experiments (20), we have shown that *D. discoideum* ribosomal 40 S and 60 S particles reconstituted in vitro from free rRNA and ribosomal proteins (or isolated from nuclei) and containing pre-17 S and pre-26 S rRNAs enter polyribosomes and synthesize polypeptides in an in vitro protein synthesis system as efficiently as cytoplasmic ribosomal subunits containing mature 17 S and 26 S rRNAs. The experiment shown in Table I (line 1) indicates that ribosomal particles newly assembled in vitro and still containing immature RNA join polyribosomes as soon as they enter the cytoplasm. Cells were removed from the growth medium, resuspended in Sorenson buffer, and labeled with $[14C]$ uracil for 8 h (to label mature ribosomal particles) and then pulsed for 30 min with $[3H]$uracil (to label newly formed ribosomal particles) before being lysed. The $^{3H}$/$^{14}C$ ratio in ribosomal subunits found free in the cytoplasm or bound to polyribosomes was the same. All of the $^{14}C$ label was in mature 17 S and 26 S rRNAs (besides 5 S and 5.8 S RNAs), whereas the $^3H$ label was partially in immature pre-17 S and pre-26 S rRNAs and partially in mature 17 S and 26 S rRNAs. The ratio of $^3H$ label in immature and mature rRNAs was the same in free ribosomal subunits and in polyribosomes and decreased in parallel during a period of chase with unlabeled uracil follow-

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* The abbreviation used is: MES, 4-morpholineethanesulfonic acid.
Ribosome Assembly and Disassembly

rRNA Maturation Occurs in Polyribosomes—When D. discoideum cells are removed from the growth medium and shaken in Sorensen buffer, polyribosomes suddenly disappear (32). All pre-existing ribosomal particles are found as 80 S monosomes or free ribosomal subunits depending on the Mg²⁺ concentration used in the sucrose gradient analysis. Protein synthesis resumes, and polyribosomes reappear after ~30 min. In this interval of time, rRNA synthesis and ribosome formation continue, presumably utilizing a pool of pre-existing ribosomal proteins (30, 33). This allows a pool of newly formed ribosomal subunits to accumulate in the cytoplasm without entering polyribosomes. In the experiment shown in Table I (line 2), cells were labeled with [³H]uracil for 16 h during growth, pulsed for 10 min with [³H]uracil at the time of starvation, and chased for 20 min with unlabeled uracil to prevent further entry of labeled ribosomal subunits into the cytoplasm. Cells were collected and lysed as soon as protein synthesis resumed. Under these conditions, all of the ³H label added to the cell suspension at the time of starvation and found in cytoplasmic 40 S and 60 S ribosomal particles 30 min later was in pre-17 S and pre-26 S rRNAs (Table I, line 2); but if pactamycin was removed 5 min before cell lysis, polyribosomes re-formed. Under these conditions (Table I, line 4), 95% of the ³H label present in free cytoplasmic ribosomal subunits was in pre-17 S and pre-26 S rRNAs, whereas ~30% of the ³H label present in ribosomal subunits derived from polyribosomes was in immature rRNAs, and 70% was in mature rRNAs. The same results were obtained when cycloheximide, a drug that blocks peptide bond formation, was added to the cell suspension instead of pactamycin. In the presence of cycloheximide, only 80 S ribosomes and no polyribosomes re-formed after 30 min of incubation, and no rRNA maturation occurred (the data are not shown since the electrophoretic pattern obtained was identical to the one summarized in Table I, line 4). These findings indicate that rRNA maturation occurs neither in free native ribosomal subunits nor even in 80 S ribosomes newly bound to mRNA (34, 35), but in polyribosomes.

| Free RNA | In free subunits | In 80 S or polyribosomes |
|----------|-----------------|------------------------|
| Pre-17 S | ³H Uracil       | In vitro             |
| Pre-26 S |                  | In vitro             |

Table I

| In ³H Uracil | In ³C Uracil |
|--------------|-------------|
| | cpm × 10⁻³ |
| | cpm × 10⁻³ |
| 1. 0 0 0 3 1 2 5 | 8 2 2 4 1 4 | 2 3 4 1 2 3 |
| 2. 0 0 0 5.9 2.5 1.8 4.2 | 10.6 2 2 4 1 4 | 3.3 7.7 8 2 2 4 |
| 3. 0 0 0 7.5 0 0 0 | 13.5 0 0 0 | 0 0 0 0 0 0 |
| 4. 0 0 0 7 0.3 1.2 2.6 | 3.2 1 2 4 1 4 | 0 0 0 0 0 0 |
| 5. 0 0 0 1 0 5.2 0.6 1.2 | 2.3 1 2 4 1 4 | 0 0 0 0 0 0 |
| 6. 0 0 0 0.2 2.4 0.1 7.1 | 0.1 4.4 1 | 0.1 14.1 8 2 2 4 |
| 7. 0 0 0 5.5 0 | 1 0 10.3 | 2.1 0 0 0 0 0 |
| 8. 0 0 0 6.4 0 0 0 | 13.1 0 0 0 | 0 0 0 0 0 0 |
| 9. 0 0 0 6.6 0 0 0 | 12 0 0 0 0 | 3.5 0 0 0 0 0 |
| 10. 0 0 0 5.2 0.1 0 0 | 10.5 0 0 0 | 0 0 0 0 0 0 |
| 11. 0 0 0 4.8 0.1 2.7 0.2 | 3 0 0 0 0 0 | 2.4 0 0 0 0 0 |
| 12. 0 0 0 0.1 4.9 0 2.9 | 7.5 0 0 0 | 2.5 0 0 0 0 0 |
| 13. 0 0 0 0.1 5.1 0.1 3.2 | 5.1 0 0 0 | 3 0 0 0 0 0 |
| 14. 0 0 0 0.2 5.2 0.2 3.4 | 4 0 0 0 0 | 3.3 0 0 0 0 0 |
| 15. 0 0 0 0.2 5.2 0.2 3 7 | 1 0 0 0 | 0 0 0 0 0 0 |
| 16. 0 0 0 2 1.1 3.5 1.8 | 3 0 0 0 | 2 0 0 0 0 0 |
| 17. 3.3 7 4.3 1 2 | 0 0 0 0 | 3.9 0 0 0 0 0 |
| 18. 0 0 0 4.1 0.1 0 1 | 8 0 0 0 | 0 0 0 0 0 0 |
| 19. 4 1.9 0.1 0 | 0 0 0 0 | 0 0 0 0 0 0 |
| 20. 0 0 0 0.5 1.8 0 3 7 | 1 0 0 0 | 0 0 0 0 0 0 |
| 21. 0 0 0 0.3 2.1 0 2 0 | 3.8 0 0 0 | 0 0 0 0 0 0 |
| 22. 0 0 0 0.3 3 1 0 0 | 8 0 0 0 | 0 0 0 0 0 0 |
| 23. 3.9 8.1 1 0 | 0 0 0 0 | 0 0 0 0 0 0 |
| 24. 0 0 0 0 3 0.8 0.1 2 3 | 0 0 0 0 | 0 0 0 0 0 0 |
| 25. 0.2 0.5 0.3 0 0 0 1 | 9 2 0 0 | 0 0 0 0 0 0 |
| 26. 0 0 0 1 1 1.1 0 | 3 0 0 0 | 2 0 0 0 0 0 |
| 27. 3.4 6.8 1 0 | 0 0 0 0 | 1 0 0 0 0 0 |
| 28. 0 0 0 0 2 4 0 | 4 0 0 0 | 0 0 0 0 0 0 |
| 29. 0 0 0 0 2 4 0 | 4 0 0 0 | 4 0 0 0 0 0 |

rRNA Maturation Requires Functioning of Immature Ribosomal Particles in Protein Synthesis—As we have previously shown (20), 40 S and 60 S ribosomal subunits isolated from nuclei containing immature rRNA synthesized polypeptides and form polyribosomes in an in vitro protein synthesis system with the same efficiency as cytoplasmic ribosomal subunits containing mature rRNA. Table I (lines 5 and 6) shows that after immature particles had functioned in protein synthesis, immature rRNA was converted into mature rRNA. If poly(A)+ RNA was omitted from the in vitro system (Table I, line 7) or if pactamycin (line 8) or cycloheximide (line 9) was added to the in vitro system, no rRNA maturation occurred.
To determine whether rRNA maturation occurs randomly during functioning of immature particles in protein synthesis or whether it requires the productive movement of ribosomes over a certain length of the mRNA molecule, poly(A)\(^+\) RNA was replaced in the in vitro protein synthesis system by the mRNA encoded by a single D. discoideum gene (AC914), the sequence of which has been determined.\(^2\) Immature ribosomal particles were incubated in the in vitro protein synthesis system in the presence of 19 amino acids, with the exclusion of a different single amino acid in different trials. Puromycin, a drug that causes dissociation of polyribosomes into free ribosomal subunits (36), was also added; at a low concentration (30 µg/ml), puromycin allows the formation of small polyribosomes and the synthesis of short polypeptides and causes recycling of the ribosomal particles between polyribosomes and the pool of free subunits even in the absence of a stop codon in the mRNA used to program the protein synthesis system.\(^3\) When methionine or leucine (the first 2 amino acids incorporated into the polypeptide encoded by AC914 mRNA) was omitted, rRNA maturation did not occur (Table I, lines 10 and 11); but when phenylalanine, the third amino acid to be incorporated, was omitted, pre-17 S RNA was converted into 17 S RNA, whereas pre-26 S RNA did not mature (line 12). The same result was observed when lysine or asparagine, the fifth and sixth amino acids to be incorporated, was omitted (Table I, lines 13 and 14) (the fourth amino acid is again leucine). Finally, if arginine, the seventh amino acid, was omitted, the conversion of pre-26 S RNA into 26 S RNA also occurred (Table I, line 15).

Immature Ribosomal Particles That Form 80 S Ribosomes but Do Not Function in Protein Synthesis Are Disassembled—If cells are washed from the growth medium and resuspended in Sorensen buffer in the presence of pactamycin (to avoid the entry of newly formed immature \(^3\)H-labeled ribosomal subunits into 80 S ribosomes) and, 30 min later, pactamycin is removed and replaced by cycloheximide, the fate of \(^3\)H-labeled ribosomal particles that form 80 S ribosomes can be followed and compared with the fate of mature ribosomal particles that have been previously labeled with \(^14\)C]uracil during growth and that have already functioned in protein synthesis. Table I (lines 16 and 17) shows that whereas the \(^14\)C-labeled particles were stable for at least 30 min, the \(^3\)H label progressively disappeared from 80 S ribosomes. All of the \(^3\)H label lost from 80 S ribosomes appeared as free pre-17 S and pre-26 S RNAs in the cytosol. Since, in the presence of pactamycin, immature ribosomal particles are stable, two conditions appear to be required for their disassembly: they must form 80 S ribosomes, but be prevented from functioning in protein synthesis.

A similar result has been obtained in the in vitro protein synthesis system programmed with AC914 mRNA. When immature \(^3\)H-labeled ribosomal particles were incubated in the presence of 19 amino acids, but in the absence of methionine, they were stable (Table I, line 18). If methionine was present, but leucine was omitted, both 40 S and 60 S particles progressively disappeared, and the label was transferred to free pre-17 S and pre-26 S RNA molecules (Table I, line 19). If the omitted amino acid was phenylalanine, 40 S particles remained stable, but 60 S particles were disassembled (Table I, line 20). Finally, if the omitted amino acid was arginine, both 40 S and 60 S particles remained stable (Table I, line 21). Thus, the signals that induce rRNA maturation and that prevent ribosomal particles engaged in protein synthesis from being disassembled appear to coincide.

\(^{2}\) A. Ceccarelli, personal communication.
\(^{3}\) G. Mangiarotti, unpublished observation.
oligonucleotides complementary to sequences present in the 5'-ends of pre-17 S and pre-26 S RNAs, but absent in mature 17 S and 26 S RNAs (38). The two oligonucleotides bind to 40 S and 60 S particles isolated from nuclei, but not to particles isolated from polyribosomes. When immature particles with the bound oligonucleotide were incubated in an in vitro protein synthesis system, they synthesized polypeptides and formed polyribosomes, but their rRNAs did not mature. The particles were progressively lost from polyribosomes and converted into free rRNA and protein molecules (Table I, lines 26 and 27). Thus, if rRNA maturation is prevented, ribosomal particles are disassembled after their entry into polyribosomes, although they are capable of synthesizing proteins.

In a complementary experiment, immature ribosomal particles with the bound oligonucleotides were incubated briefly with RNase H to digest the DNA-RNA hybrid sequences. Following this treatment, pre-17 S and pre-26 S RNAs were converted into molecules that comigrated with mature 17 S and 26 S rRNAs during electrophoresis on nondenaturing gels, although the 5'-ends of the in vitro trimmed RNA species and of those matured in vivo did not coincide, as determined by primer extension analysis (data not shown). The particles containing in vitro shorted pre-RNA molecules could form 80 S ribosomes when incubated in an in vitro protein synthesis system, but were incapable of forming large polyribosomes and of synthesizing polypeptides, due probably to the abnormal 5' termini of the rRNA molecules. However, 80 S ribosomes were stable and were not disassembled over the 30-min incubation time (Table I, lines 28 and 29). Thus, rRNA maturation mimicked in vitro was sufficient to render ribosomal particles resistant to disassembly, although they had not functioned in protein synthesis. rRNA maturation is therefore probably the event triggered by functioning of ribosomal particles in protein synthesis, and in turn, it determines the stabilization of ribosomal particles.

DISCUSSION

The results reported in this paper indicate that in D. discoideum, rRNA maturation occurs after the newly formed ribosomal subunits have entered the cytoplasm and joined polyribosomes. This finding is consistent with previous indications that in E. coli, immature rRNA can be found in 30 S and 50 S subunits present in polyribosomes (24, 39, 40); that in yeast, 40 S particles containing immature rRNA can be found in the cytoplasm (25); and that in D. discoideum, immature ribosomal subunits can form polyribosomes in an in vitro protein synthesis system (20). The fact that in yeast and in other eukaryotes, immature rRNA has not been detected in polyribosomes does not exclude the possibility that in these organisms, immature ribosomal subunits enter polyribosomes since if rRNA maturation occurs immediately after this event, immature rRNA would not accumulate at a detectable level.

The entry of immature ribosomal subunits into polyribosomes is not a casual, but an obligatory step in their metabolism. Experiments carried out both in vivo and in vitro indicate that if newly formed ribosomal particles do not function in protein synthesis, their rRNAs do not mature. The use of a protein synthesis system programmed by a single species of cellular mRNA has shown that rRNA maturation is subordinated to the ability of the ribosomal particles to perform specific steps in the process of peptide synthesis. 40 S particles must become part of an 80 S ribosome capable of catalyzing the synthesis of a first peptide bond or of undergoing a first translocation step, and 60 S particles must participate in the synthesis of a peptidyl chain at least 6 amino acids long. Since these results have been obtained by testing only one mRNA, they cannot be generalized, but they indicate that rRNA maturation is not triggered generically by the involvement of ribosomal particles in the process of protein synthesis, but by specific signals, which arise when the interaction of ribosomal particles with the other components of the protein synthesis machinery has reached certain stages, for the precise definition of which further experiments will be needed.

Immature ribosomal particles have a less stable conformation than mature particles, as shown by the fact that they are more easily unfolded in unfavorable salt conditions. However, as long as immature particles remain as free subunits either in the cell cytoplasm or in an in vitro protein synthesis system, they survive as such. When they enter polyribosomes, they must perform some steps in protein synthesis, otherwise they are disassembled into free immature rRNA and ribosomal proteins. The use of oligonucleotides complementary to the 5'-ends of pre-17 S and pre-26 S RNAs to block or to mimic their maturation in vitro has shown that the initial event triggered by functioning of ribosomal subunits is the maturation of their RNAs and that it is this event that leads to the stabilization of their structure.

The disassembly of newly formed ribosomal particles appears to be a relatively frequent event in vivo. The possibility of causing misassembly of the particles in the in vitro reconstitution process has allowed us to show that ribosome misassembly may be a cause of their lack of ability to function in protein synthesis and of the consequent persistence of immature rRNA. This in turn leads to the disassembly of the particles. If the frequency of in vivo disassembly of newly formed particles is taken as an indication of the frequency of incorrect ribosome assembly, the phenomenon appears to occur relatively often in D. discoideum. It is thus reasonable to presume that cells have evolved a mechanism to prevent the consequences of these mistakes. Entry of a non-functional or poorly functional particle into a polyribosome will lead to its sequestration as a nonfunctional structure. If this event were frequent, cells would be severely depleted of mRNA molecules and functional ribosomes and might accumulate significant amounts of incomplete polypeptide chains. Disassembly can eliminate misassembled ribosomal particles and lead to recycling of their components for the assembly of other ribosomes.

On the other hand, if ribosomal particles were subjected to disassembly before entering polyribosomes, they could not be tested for their ability to function in protein synthesis. Thus, it is not surprising that their disassembly can occur only in polyribosomes.

If our hypothesis is correct, there are at least two roles that rRNA processing in several steps, rather than in a single step, might have in ribosome formation. First, the extra sequences present in immature rRNA facilitate the assembly of ribosomal proteins on the RNA molecules (20, 23). However, this process leads to the formation of a relatively unstable reversible structure. rRNA maturation then occurs, leading to the conversion of the particle into an irreversible complex only if the initial immature rRNA-protein complex proves to be structurally correct and functional in protein synthesis. Since rRNA maturation and ribosomal particle stabilization can be reproduced in vitro in a protein synthesis-dependent process, dissection of the in vitro system should allow the determination of the factors involved in the quality control mechanism of ribosome assembly.

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