# A PROPOSED ROLE FOR 5S RIBOSOMAL RNA

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Received November 30,1972

SUMMARY
Cytoplasmic ribosomes show protein-synthesizing activity with degraded large and small rRNA's, but only if 5S RNA is intact.

The biological specificity and function of ribosomal proteins have been partially established.[1] These proteins interact sequentially and specifically with rRNA to form functional ribonucleoprotein particles.[1-4] Further, at least some of the ribosomal proteins are necessary for the biological activity of the ribosomes.[5-7] As yet, however, no role has been established for the 5S RNA of ribosomes.

Recent experiments[8,9] show a ribonuclease associated with the cytoplasmic ribosomes (87S) of Euglena gracilis. This ribonuclease is active only when the ribosomes are dissociated into subunits at low $Mg^{+2}$ ion concentrations and is not removed with 2.0 M salt ($NH_4Cl$ or KCl). This activity, however, is inhibited readily by heparin (10 µg/10 mg ribosomes).[9] In the present study, this system is used to provide evidence that the 5S RNA is degraded at the time protein synthesis terminates and therefore 5S RNA may be essential for the activity of the 87S ribosomes.

## MATERIALS AND METHODS

A bleached strain (SM-L1) of E. gracilis was grown in 6 liters of defined medium[10] with 0.2 M ethanol[11] as carbon source as

described.[9] Cells were harvested when they reached a density of 4 X $10^6$ per ml., washed twice with KTM 15 buffer (25 mM KCl, 50 mM Tris-HCl, 15 mM $MgCl_2$, pH 7.6) containing 0.25 M sucrose and suspended in two volumes of buffer. The RNase inhibitors heparin[12] (Sigma, lithium salt, at 500 µg/ml) and a ribonuclease-specific protein fraction from rat liver[13] (1 mg protein/ml) were added to cells prior to grinding with glass beads.[9,14] The resultant homogenate was centrifuged at 15,000 X g for 15 min. The supernatant was mixed with 10% sodium deoxycholate in water to obtain a final concentration of 1% deoxycholate. The treated supernatant (2.3 ml) was layered over 1.0 ml of 2.0 M sucrose (containing KTM15) and centrifuged in an SB 405 rotor of an International Ultracentrifuge, Model B-60, at 405,000 X g (max.) for 120 min at 0-2°C as described.[9] The resultant polysomal pellet was rinsed 3-4 times with KTM.2 buffer (25 mM KCl, 50 mM Tris-HCl, 0.2 mM $MgCl_2$, pH 7.6) and suspended in the same buffer containing 0.25 M sucrose and 25 µg/ml heparin. Such polysomes completely dissociate into 47S and 68S ribosomal subunits when incubated for 5 hr in an ice bath in this low $Mg^{++}$ buffer (KTM.2). Ribosomal subunits so prepared were used in the following experiments.

## RESULTS AND DISCUSSION

Kinetics of rRNA degradation: Incubation of the ribosomal subunits at room temperature in the absence of heparin results in degradation of both the 20S and 25S RNA's[9] as a function of time (Fig. 1). In contrast, ribosomal subunits incubated for as long as 60 min in the presence of heparin (Fig. 1F) are not degraded and appear like the unincubated controls (Fig. 1A). Gel analyses (Fig. 1A,B,C,D,F) also show the initial presence of 5-6S RNA (hereafter referred to as 5S RNA) and further reveal that this

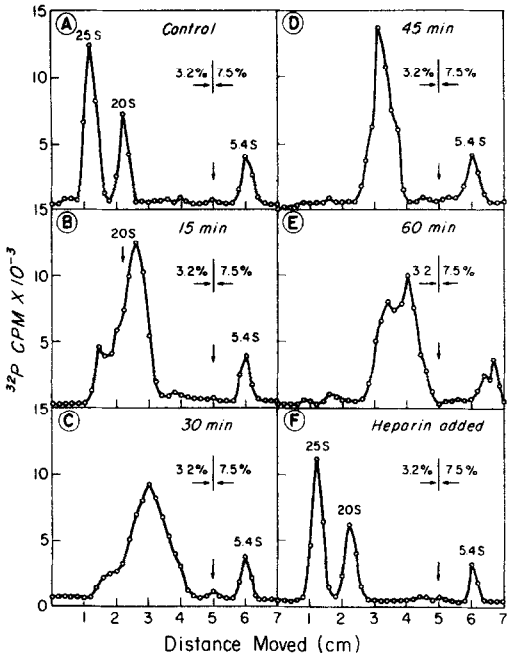


Fig. 1. Tandem-gel electrophoresis of Euglena ribosomal RNA. Cells were grown for 48 hr with 1 mCi of $H_3{}^{32}PO_4$ per 6 liter culture. Ribosomal subunits were prepared as described in the text and incubated with or without added heparin (10 µg/ml). At the end of incubation, aliquots of ribosomes containing 60,000 counts/min (100 µg rRNA = $10^6$ counts) were mixed with 10% sodium dodecyl sulfate (SDS) to a final concentration of 2% SDS and immediately layered on the gels. The gels (7mm diameter) consisted of 5 cm of 3.2% polyacrylamide and 2 cm of 7.5% polyacrylamide. Electrophoresis was done at 4°C for 3.5 hr at 5 mA/gel as described.[15] The gels were sliced into 2 mm-thick slices. Each slice was dissolved in 0.2 ml of 24% $H_2O_2$ in a scintillation vial and five ml of Aquasol (New England Nuclear) was added. The efficiency of counting was 25-30%. Sedimentation values were calculated with E. coli rRNA. (A) Control, no incubation; (B-E) incubated without heparin for 15 to 60 min; (F) incubated with heparin for 60 min.

RNA is undegraded for as long as 45 min (Fig. 1B-D). At 60 min, however, the 5S RNA appears degraded (Fig. 1E).

It is possible that the apparently undegraded 5S RNA obtained from ribosomal subunits incubated up to 45 min in the absence of heparin represents various small degradation products of the larger rRNA's. To check this possibility, the 5S RNA was eluted from the 7.5% gel portions with 5 mM Tris-HCl (pH 7.4) buffer and subjected to electrophoresis again (Fig. 2). The electrophoretic patterns

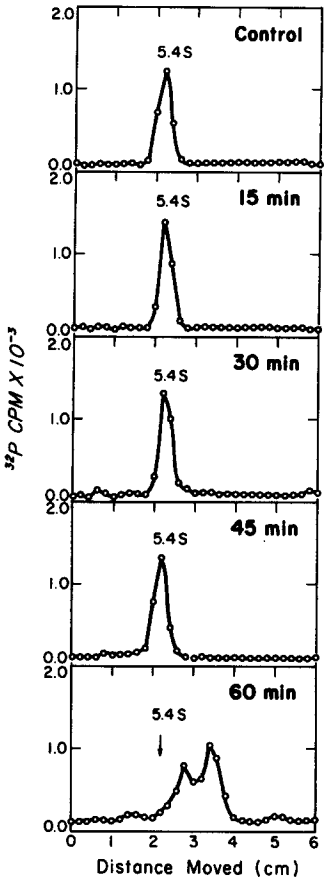


Fig. 2. Electrophoresis of 5S RNA. Polyacrylamide gel portions corresponding to 5.4 cm to 7.0 cm (Fig. 1A-E) were cut out and the radioactive RNA component was eluted from these as described.[16] The eluted RNA samples were concentrated[16] and layered on 8.0% polyacrylamide gels. Electrophoresis was done for 4 hr at 5 mA/gel. Other details were as described in Fig. 1.

show that all the eluted samples contained molecules of uniform molecular weight up to 45 min of incubation in the absence of heparin.

Biological activity: This system, with its observed protection of 5S RNA during the course of rRNA breakdown, provides a unique opportunity to examine the functions of rRNA and 5S RNA in protein synthesis. Specifically, one can ask whether or not ribosomes with degraded 5S RNA are capable of protein synthesis. In vitro

protein-synthesizing experiments were, therefore, run with ribosomal subunits incubated for various times in the absence of heparin. These experiments show that ribosomes retain their protein-synthesis capabilities when breaks are made in their large rRNA's. Further, they only lose their protein-synthesizing ability at a time when their 5S RNA is degraded (Table I). Protein synthesis has been recorded in several systems using ribosomes containing degraded rRNA,[20-24] but the status of 5S RNA has not been reported in any of these systems. The reduced protein-synthesizing activity coincident with 5S degradation (Table I) is not due to any overall size change in the ribosomal subunits during incubation for separate experiments show little change in the S-values of the ribosomal subunits over 6 hrs of incubation in the absence of heparin. Other experiments support the timing of the loss of protein synthesis potential observed in Table I. Specifically, incubation of ribosomal subunits for up to 45 min without heparin does not inhibit the ability of these subunits to form mono- and poly-ribosomes as detected by sucrose-gradient centrifugation.

5S RNA: The precise role of 5S RNA in ribosomal function is, so far, unknown. In the experiments reported here (Table I), a requirement for intact 5S RNA during initiation of protein synthesis is indicated. It is therefore practical to determine if 5S RNA is required during the formation of the 87S initiation complex.

Specifically, one can ask whether or not the initiation complex will form when degraded 5S RNA is present in the large subunit. Accordingly, ribosomal subunits were incubated for 60 min without heparin (5S RNA degraded, Figs. 1E, 2) and added to a cell-free system containing a labelled Euglena mRNA. As seen in Fig. 3, the mRNA attaches to the 48S ribosomal subunit containing degraded rRNA, but the 87S initiation complex does not form. As seen in Fig. 4,

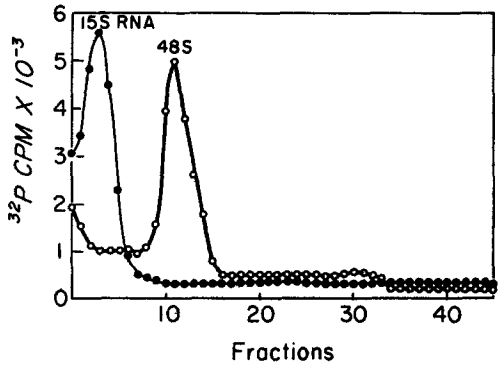


Fig. 3.

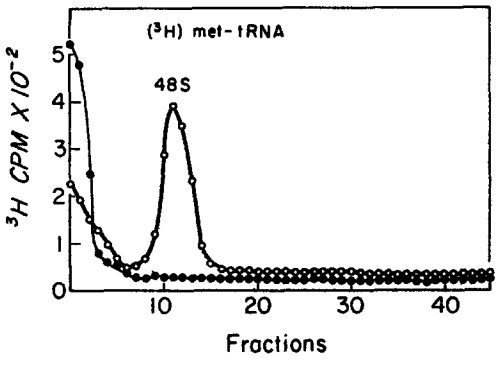


Fig. 4.

Fig. 3. Non-formation of the 87S initiation complex using ribosomal subunits incubated for 60 min without heparin. Unlabeled ribosomal subunits were incubated for 60 min. at room temperature (without heparin), then mixed with 10 µg/ml heparin to inhibit further RNase activity, and added to an in vitro system (Table I) containing $^{32}P$-labeled 15S mRNA.[16] The reaction was run for 30 min as described in Table I except that unlabeled amino acids were used. Samples containing 50 µg of RNA were layered on 3.4 ml exponential[25] sucrose-gradients (10-34%) and centrifuged at 405,000g for 2 hr. Forty-five fractions of 3 drops each were collected and counted with 5 ml Aquasol (New England Nuclear), o—o—o. In a separate tube, labeled 15S mRNA alone was centrifuged on a 10-34% exponential gradient and used as a marker, •—•—•.

Fig. 4. Attachment of ($H^3$)Met-tRNA to the 48S subunit. Assays were run using ribosomal subunits first incubated for 60 min without heparin and then mixed with 10 µg/ml heparin to inhibit further RNase activity. 15S mRNA[16] was added as described in Fig. 3 except that unlabeled mRNA was used. Unfractionated tRNA was isolated[26] from the cytoplasmic fraction of E. gracilis and amino-acylated with $H^3$-methionine (New England Nuclear, 5 mCi/mM) using E. coli enzymes.[27] When approximately 3,000 $H^3$ CPM were added to the above assay system and centrifuged on a 10-34% exponential sucrose-gradient as described in Fig. 3, the $H^3$ counts migrated to the 48S region of the gradient, o—o—o. As a control, $H^3$-labeled initiator tRNA alone was centrifuged on a 10-34% exponential gradient in a separate tube. The label in this case remained at the top of the gradient, •—•—•.

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initiator tRNA also attaches to the 48S subunit indicating that the 48S component of Figure 3 is a ribosomal subunit - mRNA - aminoacyl tRNA complex. Yet, as further indicated in Fig. 4, the next step, i.e., the attachment of the large (68S) subunit to this complex, does not occur. Formation of the 87S initiation complex involves

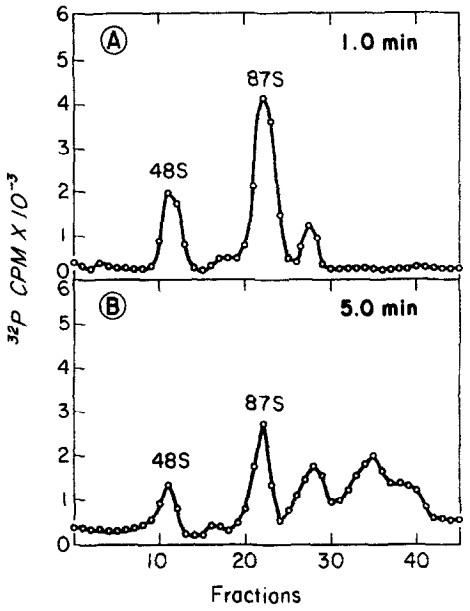


Fig. 5. Attachment of 68S subunits containing intact 5S RNA to the initiation complex. Ribosomal subunits were prepared as described in the text with heparin present at all steps. Assays were started with only the 48S subunits and $^{32}P$-labeled 15S mRNA using conditions as in Table I except that amino acids were not labeled. After 30 min, 0.1 ml of 68S subunits (equivalent to 50 µg RNA), previously incubated for 45 min without heparin, were mixed with 10 µg/ml heparin to inhibit further RNase activity and then added to the reaction mixture. Samples (0.1 ml) were taken after 1 min. (A) and after 5 min. (B) and centrifuged on 3.4 ml exponential gradients as in Fig. 3. Gradients were analyzed as in Fig. 3.

association of the 68S ribosomal subunit with a 48S ribosomal subunit to form an 87S ribosome - mRNA - aminoacyl tRNA complex. 5S RNA is an integral part of the 68S ribosomal subunit. The failure of 68S ribosomal subunits containing degraded 5S RNA to form into 87S initiation complexes suggests that 5S RNA is essential to formation of functional 87S ribosomes.

The above observation that the 87S complex does not form in the presence of degraded 5S RNA suggests a control experiment that determines if the 87S initiation complex will form when intact 5S RNA is still present in the large subunit containing degraded 25S rRNA (i.e., after 45 min. incubation in the absence of heparin,

Table I. Protein synthesis.

| Description | CPM/0.2 ml | % Activity |
|---|---|---|
| Control | 21,200 | 100 |
| 15 min incubated | 20,650 | 97 |
| 30 min incubated | 18,960 | 89 |
| 45 min incubated | 18,520 | 87 |
| 60 min incubated | 2,310 | 11 |
| 60 min incubated with heparin | 19,750 | 94 |

Aliquots of ribosomal subunits from Fig. 1 were mixed with 10 µg/ml of heparin to inhibit further RNase activity and used for these experiments. A 15S RNA was isolated from Euglena total RNA as previously described[16,17] and used as messenger RNA. Ribosomes equivalent to 100 µg RNA were used in each case. Protein-synthesis assays were as described earlier[18,19] except that 1.0 µCi of $C^{14}$ amino acid mix (New England Nuclear 1.8 µCi per mM) was added to each tube. Samples were heated with 5% TCA for 30 min in order to solubilize all the $^{32}P$ counts in RNA. Tubes without added ribosomes gave 900-1200 counts/min in this system. Other details were as described in Fig. 1 and in the text.

Fig. 1D, 2). Accordingly, subunits were incubated for 45 min. without heparin and added to a cell-free system containing a labelled Euglena mRNA. As seen in Fig. 5, the 87S complex is formed within 1 min. and polysomes are formed by 5 min. Therefore, the 87S complex forms when intact 5S RNA is still present in the large subunit even though the 25S rRNA of the large subunit is degraded.

In summary, our results show that the ability to form the initiation complex is lost after RNase treatment that is sufficient to hydrolyse 5S RNA. One possibility is that the hydrolysis of 5S RNA is coincident in time with another event, e.g., the advanced destruction of rRNA or the loss of certain ribosomal proteins, which in turn is responsible for the inability of the initiation complex to form. If so, the hydrolysis of 5S RNA can serve as a time-marker for the critical event. Another possibility is that intact 5S RNA itself is required for the attachment of the large ribosomal subunit to the initiation complex.

This work was supported by grant HD 03163 from the National Institute of Child Health and Human Development. A portion of these data has been presented elsewhere in abstract form.[28]

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