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Activity of Ribosomes Containing 5S RNA with a Chemically Modified 3'-Terminus*

(*B. stearothermophilus*/peptidyl-tRNA/periodate/morpholine derivative/protein synthesis)

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ABSTRACT Ribosomes containing 5S RNA in which the 3'-terminus has been modified chemically, either by periodate oxidation or by conversion of the terminal ribose to a morpholine derivative, are fully as active in peptide-bond formation and polypeptide synthesis as those ribosomes with unmodified 5S RNA. These results exclude models for protein synthesis in which peptidyl-5S RNA is formed as an intermediate.

We have shown that 5S RNA is essential for the structure and activity of the large ribosomal subunit (1). However, no specific role in ribosomal function has yet been attributed to 5S RNA except that it is required for the binding of several ribosomal proteins. Raacke (2) has proposed a model for protein synthesis in which peptidyl-5S RNA is formed as an intermediate during peptide-bond formation. According to this model, the nascent polypeptide becomes esterified to the 3'-hydroxyl group of the terminal nucleoside of 5S RNA by transfer from peptidyl-tRNA, then is transferred to aminoacyl-tRNA. We have tested this proposal by making use of reconstitution of the 50S subunit that is dependent on added 5S RNA (1). The activity of chemically modified 5S RNA (in its 3'-terminal nucleoside) was analyzed in this system. Our results, reported here, indicate that the detailed model proposed by Raacke (2), and probably any other model that involves formation of peptidyl-5S RNA, is incorrect.

MATERIALS AND METHODS

Ribosomes were prepared from *Bacillus stearothermophilus* as described (1), except that the ribosomes were washed by sedimentation from the cell extract (S 30) through a layer of 1.1 M sucrose–0.5 M NH_4Cl–10 mM $MgCl_2$–20 mM Tris·HCl (pH 7.4)–6 mM 2-mercaptoethanol (3). Protein and RNA fractions were prepared from these washed 70S ribosomes by treatment with 4 M urea–2 M LiCl, and both fractions were freed of 5S RNA as described (1).

Periodate Oxidation of 5S RNA. Purified 5S RNA (1) was treated with $NaIO_4$ as follows. To 10 A_{260} units of 5S RNA in 0.25 ml of 0.1 M NaOAc (pH 5.0) was added 12 μl of 0.1 M $NaIO_4$ (120-fold molar excess of periodate). After incubation for 1 hr at 23°C in the dark, 0.5 ml of ethanol was added and the RNA was allowed to precipitate for 30 min at −20°C and recovered by centrifugation. The RNA was washed by dissolving it in 0.1 M NaOAc and reprecipitating it with ethanol. The extent of oxidation of the terminal ribose by periodate was determined by chromatography on aminoethylcellulose (ref. 4 and Johnson, T., personal communication). This analysis is based on the fact that the dialdehyde group formed by periodate oxidation of the terminal ribose residue of RNA forms a covalent complex with the amino groups of the resin. Identical samples of periodate-treated and untreated 5S RNA in 0.3 M KCl were applied to identical aminoethylcellulose columns (equilibrated with 0.3 M KCl at pH 7.0) and allowed to remain in contact with the resin for 2 hr at 2°C. The columns were then washed with 0.3 M KCl, and eluted with 1 M KCl. The amount of RNA eluted from the column by 1 M KCl provides a measure of the amount of unoxidized RNA, since RNA with the dialdehyde terminus remains bound to the column. This analysis indicated that 92% of the periodate-treated 5S RNA had the dialdehyde group.

Methylamine–Borohydride Reaction. Periodate-oxidized and untreated 5S RNA were carried through the following procedure in parallel. To 10 A_{260} units of 5S RNA in 0.2 ml of 0.1 M $NaBO_3$ (pH 9.0) was added 0.1 ml of 2.9 mM [^{3}H]methylamine·HCl (New England Nuclear Corp.; 34 Ci/mol, methyl labeled) in ethanol. After 1 hr of incubation on ice, 0.1 ml of 0.05 M $LiBH_4$–0.1 M $NaBO_3$ (pH 9.0) was added and another 1-hr incubation was performed. The reaction was then terminated by the addition of 5 μl of 0.1 M EDTA, followed by 0.4 ml of 0.5 M NaOAc (pH 3.5) (5). After 5 min on ice, during which effervescence was observed, 2 ml of ethanol was added. The precipitated RNA was washed five times by redissolving it in 0.5 ml of 2% KOAc (pH 5)–0.05 M methylamine, and reprecipitating it with ethanol. After this procedure, the periodate-oxidized RNA contained 0.80 mol of [^{3}H]-methylamine per mol of 5S RNA, while the unoxidized RNA contained 0.02 mol of [^{3}H]methylamine per mol of 5S RNA.

RESULTS AND DISCUSSION

Ribosomes were reconstituted with 5S RNA-free RNA (23S and 16S RNAs) and protein (a mixture of 50S and 30S proteins) fractions (1). Particles were produced in the absence of 5S RNA ([−5S] particles), in the presence of periodate-treated 5S RNA ([+5S-IO_4] particles), and untreated 5S RNA ([+5S] particles). The amount of 5S RNA added to the reconstitution mixture was 1 mol/mol of 23S RNA. Reconstituted particles were recovered by centrifugation and their activities were determined as described in Table 1. [−5S] Particles have greatly reduced activity in both poly(U)-dependent polyphenylalanine synthesis (9% of control [+5S]

* Paper XIV in the series *Structure and Function of Bacterial Ribosomes*. Paper XIII in this series is ref. 1.

TABLE 1. *Activity of particles reconstituted with periodate-treated 5S RNA*

	Poly(U) assay (cpm)	Peptidyl transferase assay (cpm)
Reconstituted [−5S]	386	1,000
[+5S–IO_4^-]	4,680	3,800
[+5S–untreated]	4,300	3,530
Native 70S	11,500	10,300

Reconstituted particles (consisting of "50S" and "30S" particles) were assayed for poly(U)-directed synthesis of polyphenylalanine (8) in the presence of excess added 30S subunits, and for the formation of fMet-puromycin from fMet-tRNA (peptidyl-transferase assay) in the presence of 33% methanol. The transferase assay was performed essentially as described by Miskin *et al.* (9). The reaction was initiated by the addition of 50 μl of methanol containing 1 mM puromycin to each 100-μl assay mixture, which contained 60 mM Tris·HCl (pH 8.1 at 0°C)–0.4 M KCl–20 mM $MgCl_2$–50,000 cpm of [^{35}S]fMet-tRNA (1). After incubation for 10 min at 0°C, the reaction was terminated by the addition of 10 μl of 10 N KOH and incubation at 37°C for 10 min. The mixture was then extracted with 1.5 ml of ethyl acetate after the addition of 1 ml of 1 M sodium phosphate (pH 7.0) and 1 ml of the ethyl acetate layer was removed for counting. In both assays each sample contained 1.3 A_{260} units of reconstituted or native (undissociated) ribosomes, which contained 1.0 A_{260} unit of 50S subunits or reconstituted "50S" particles. In addition, each poly(U) assay sample contained 1.0 A_{260} unit of *Escherichia coli* 30S subunits. Blank values, obtained in the absence of 50S subunits, have been subtracted to obtain the data shown. The blank values were 279 cpm for the poly(U) assay and 81 cpm for the peptidyl-transferase assay.

particles) and the peptidyl-transferase assay (28% of control [+5S] particles). There is no significant difference in either assay between [+5S–IO_4] particles and [+5S] particles.

Analysis of the [+5S–IO_4] particles by aminoethylcellulose chromatography after phenol extraction, however, revealed that the dialdehyde group was no longer present in the 5S RNA after reconstitution. Since essentially all of the periodate-treated 5S RNA added to the reconstitution mixture is incorporated into particles, the absence of the dialdehyde group after reconstitution must be due to its loss from the 5S RNA during reconstitution, undoubtedly by the well-known amine-catalyzed β-elimination reaction (6, 7). The presence of Tris in the reconstitution buffer may be responsible in this case. Thus, the [+5S–IO_4] particles contain 5S RNA that has lost its 3′-terminal nucleoside residue altogether, and presumably retains a phosphate at the 3′(2′)-position of its original penultimate nucleoside.

Though it is unlikely that such a modified 5S RNA would retain full activity if the peptidyl-5S RNA model were correct, a more convincing test of the proposal was performed by the use of 5S RNA with a stable modification. This was accomplished by treatment of periodate-oxidized 5S RNA with methylamine, followed by reduction with $LiBH_4$. As shown by Khym (7), using adenosine 5′-phosphate, this procedure converts the terminal ribose to a 5-hydroxy-4-methyl-2-hydroxymethylphosphate morpholine derivative, in which the 3′-hydroxyl involved in the Raacke model is missing and the stereochemical structure of the terminal residue is considerably altered (Fig. 1).

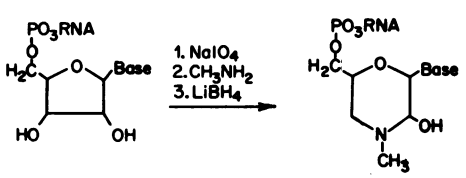

FIG. 1. Chemical modification of the terminal residue of RNA by periodate–methylamine–borohydride treatment. The structure of the product is according to Khym (7).

Periodate-oxidized, as well as unoxidized, 5S RNA was treated with methylamine and borohydride, as described in *Methods*. The activity of particles reconstituted as described above, in the presence of these preparations of 5S RNA, is shown in Table 2. There is no significant difference between the activity of ribosomes that contain untreated 5S RNA and those with periodate–methylamine–borohydride-treated 5S RNA, in either the poly(U)-dependent polyphenylalanine synthesis or peptidyl-transferase assay.

The particles reconstituted with periodate–methylamine–borohydride-treated 5S RNA were analyzed to determine whether the [^{3}H]methylamine, which was incorporated into the morpholine end-group of the modified RNA, is present. The results of sucrose gradient sedimentation are shown in Fig. 2. The peak of radioactive material coincides with the A_{260} peak of reconstituted 50S particles. At the peak, the molar ratio of [^{3}H]methylamine to 23S RNA is 0.6. Though this is lower than the expected value of 0.8, the difference is accounted for by the fact that these reconstituted particles have only 80% of the theoretical amount of 5S RNA, as determined by analysis of stained polyacrylamide gels.

These experiments demonstrate that 5S RNA retains full activity when its 3′-terminal residue is removed or when its ribose is converted to a stable morpholine derivative. These results are inconsistent with the model proposed by Raacke (2). It is highly unlikely that peptidyl-5S RNA is involved as an obligatory intermediate in protein synthesis.

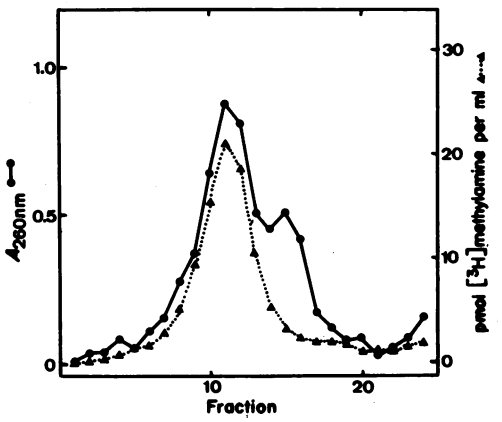

FIG. 2. [^{3}H]Methylamine content of reconstituted particles. An aliquot (6.5 A_{260} units) of particles reconstituted in the presence of periodate–[^{3}H]methylamine–borohydride-treated 5S RNA was analyzed by sedimentation in a 5–20% sucrose gradient containing 10 mM Tris·HCl (pH 7.4)–60 mM NH_4Cl–0.3 mM $MgCl_2$ in a Spinco SW50 rotor at 50,000 rpm for 90 min. 3-Drop fractions were collected from the bottom of the tube into 0.8 ml of H_2O. After determination of A_{260}, an aliquot of each fraction was removed for determination of ^{3}H in a liquid scintillation counter. Sedimentation was from *right* to *left*.

TABLE 2. *Activity of particles reconstituted with periodate–methylamine–borohydride-treated 5S RNA*

	Poly(U) assay (cpm)	Peptidyl-transferase assay (cpm)
Reconstituted [−5S]	377	1,640
[+5S–IO_4–CH_3NH_2–$LiBH_4$]	2,100	3,410
[+5S–CH_3NH_2–$LiBH_4$]	1,870	2,660
[+5S–untreated]	2,010	3,310
Native 70S	9,240	15,600

Assays were performed as described in Table 1. The blank values, which were subtracted to obtain the data shown, were 273 cpm for the poly(U) assay and 72 cpm for the peptidyl-transferase assay.

Further evidence is provided by the residual peptidyl-transferase activity of particles reconstituted in the absence of 5S RNA (1), especially as shown in Table 2. These [−5S] particles, which have about half the peptidyl-transferase activity of [+5S] particles, were found to contain no detectable 5S RNA (i.e., less than 1% of the amount present in [+5S] reconstituted particles) by analysis of stained polyacrylamide gels. The residual activity of [−5S] particles in the peptidyl-transferase assay is observed consistently (1), but is generally between 10 and 25% as compared to [+5S] particles. Therefore, though peptidyl-transferase activity is strongly dependent on the presence of 5S RNA, the ribosome is able to make peptide bonds to some (reduced) extent in the absence of 5S RNA. It is unlikely, therefore, that 5S RNA is involved in the reaction in so direct and obligatory a manner as the formation of peptidyl-5S RNA would require.

A similar conclusion has been reached independently by Erdmann and coworkers (Erdmann, V. A., personal communication).

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