RESISTANCE OF E. COLI RIBOSOMAL 5S RNA TO DEGRADATION BY RIBONUCLEASE IN RECONSTITUTED RIBOSOMAL PARTICLES

Bernard G. Forget* and Max Reynier

Centre de Biochimie et de Biologie Moléculaire, C.N.R.S., Marseille, France

Received February 23, 1970

SUMMARY

Radioactive E. coli ribosomal 5S RNA was reattached to non-radioactive larger ribosomal subunits from which the 5S RNA had previously been removed by treatment with lithium chloride. After exposure of such reconstituted ribosomal particles to high concentrations of RNase T1, most of the radioactive 5S RNA remains acid precipitable. Approximately half of this 5S RNA remains attached to particles sedimented at 100,000 g and appears to be unaltered intact 5S RNA when analysed by column chromatography, acrylamide gel electrophoresis, in the presence of 7M urea, and by oligonucleotide fingerprinting techniques.

When E. coli ribosomal subunits are exposed to 2M LiCl, approximately 70-80% of the 5S RNA and approximately 50% of the ribosomal proteins become dissociated from the larger, 50S, ribosomal subunits (1). If the sedimented LiCl treated particles are resuspended in their corresponding 100,000 g supernatant (containing LiCl split proteins and 5S RNA to which are added equimolar amounts of radioactive 5S RNA) and the mixture is dialysed against a buffer containing KCl, $MgCl_2$ and Tris-HCl, the ribosomal proteins and approximately 50% of the radioactive 5S RNA become reassociated with the larger ribosomal subunits. The sedimentation coefficient of the latter is approximately 27S after LiCl treatment and approximately 48S after reconstitution (1-3). Such reconstituted particles have approximately the same RNA/protein composition as naturally occurring 50S ribosomal subunits, but they are not active in a cell-free, protein synthesizing system. However, if KCl is removed from the suspension of particles by dialysis, and the particles are preheated to 37°C in the presence of mercaptoethanol, binding of tRNA to the particles can occur (2). We used this

*Present address: Hematology Research, Children's Hospital Medical Center, Boston, Mass.

system to study the susceptibility to attack by RNase of 5S RNA which is bound to ribosomal particles.

MATERIALS AND METHODS

Ribosomal subunits, prepared from the RNase I^-_{10} strain of E. coli, were exposed to 2M LiCl for 15 hours at 4°C, sedimented at 100,000 g and the fractions stored in liquid nitrogen (1-3). Particles were resuspended in their corresponding split protein solution, and reconstituted by dialysis for 24 hours at 4°C against a solution of 0.1M KCl, 0.01M $MgCl_2$ and 0.005M Tris-HCl, pH 7.6, (KMT buffer). In some cases, reconstituted particles were then dialysed against a solution of 0.01M $MgCl_2$ and 0.005M Tris-HCl, pH 7.4 (4) then heated to 37°C for 1 hour in the presence of 0.006M mercaptoethanol (2,3). Mixtures of 30S and 50S subunits were used throughout, since reconstituted particles do not associate to form 70S-like ribosomes (2). Radioactive 5S RNA was prepared from ^{32}P-labeled E. coli EA_2 or K_{12} S20SOS cells (5) by phenol extraction of whole cells (6) or purified ribosomes (5), and was purified by Sephadex gel filtration (7) and/or methylated albumin Kieselguhr (MAK) column chromatography (8).

Reconstituted particles were sedimented at 100,000 g for 5 hours, resuspended in KMT buffer and exposed for 30 minutes at 37°C to T1 or pancreatic RNase, at various enzyme:substrate (E/S) ratios. Most experiments were done using 5 µg T1 RNase/100 µg total RNA (E/S: 1/20) and 2 µg pancreatic RNase/100 µg total RNA (E/S: 1/50).

The 5S RNA remaining attached to the RNase-treated particles was studied after sedimentation of the particles at 100,000 g for 5 hours. The RNA was extracted with phenol at 4°C, and fractionated by Sephadex gel filtration (7), MAK column chromatography (8), or acrylamide gel electrophoresis (6,9) in the presence of 7M urea (3). The 5S RNA, thus purified was completely digested with RNase and the digests fractionated by electrophoresis to obtain two dimensional oligonucleotide fingerprints of the RNA (10,11).

RESULTS

Figure 1 shows the percentage of radioactive 5S RNA remaining acid

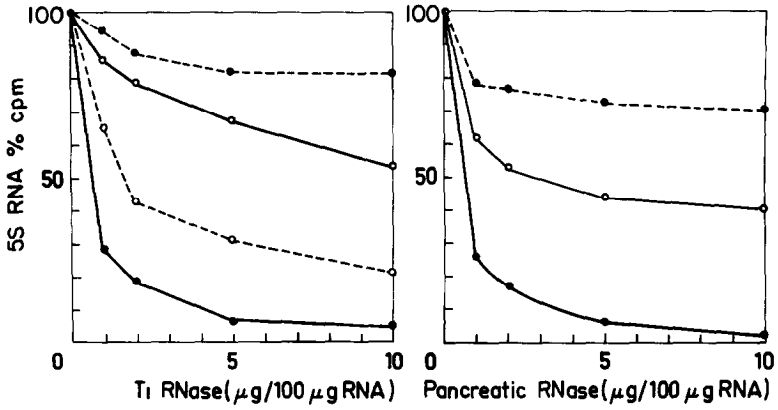

Figure 1. Susceptibility to degradation by RNase of 5S RNA bound to reconstituted ribosomal particles.

Radioactive 5S RNA was added to LiCl stripped ribosomal particles resuspended in their own split proteins and the mixture dialysed against 0.1M KCl, 0.01M $MgCl_2$ and 0.005M Tris HCl, pH 7.6 (KMT buffer) for 24 hours at 4°C. The particles were then sedimented, resuspended in KMT buffer and exposed to varying quantities of pancreatic or T1 RNase for 30 minutes at 37°C. An equal volume of cold 10% trichloracetic acid (TCA) was then added to the suspensions, and the precipitate counted on filters. The total RNA content of the suspension (ribosomal RNA + 5S RNA) was used to determine the enzyme/substrate ratio (μg RNase/100 μg RNA). Resistance of 5S RNA to degradation is expressed as 100 x TCA precipitable CPM of RNase treated suspension/TCA precipitable CPM of a control suspension containing the same initial number of CPM, but not exposed to RNase: 5S RNA-% CPM recovered.

● - - - - - ● 5S RNA bound to reconstituted particles which after reconstitution were preincubated at 37°C in the presence of mercaptoethanol.

O ———— O 5S RNA bound to reconstituted particles.

O - - - - - O 5S RNA bound to reconstituted particles which after exposure to RNase were sedimented at 100,000 g prior to TCA precipitation.

● ———— ● Control: 5S RNA not bound to particles, but free in a suspension of reconstituted particles in KMT buffer.

---

precipitable after exposure of reconstituted LiCl-treated ribosomal particles to various concentrations of T1 and pancreatic RNase. In conditions where 5S RNA, unattached to ribosomal particles, is completely degraded, the 5S RNA attached to reconstituted particles remains 50% to 70% acid precipitable. When the particles are preheated to 37°C in the presence of mercaptoethanol, the 5S RNA is even more resistant (70% to 85%) to degradation by RNase. The preheating of the particle must confer to it a more physiological conformation

since this procedure restores the ability of tRNA to bind to the particles (2,3). However, when the RNase-treated particles are first sedimented at 100,000 g before assay of acid precipitable radioactivity, they contain only approximately 50% as much acid precipitable radioactivity as the non-sedimented particles similarly treated. This suggests that the reconstituted LiCl particles are a heterogeneous mixture of particles, some of which are more extensively degraded by RNase, so that they will no longer sediment at 100,000 g or will be further disrupted during sedimentation and liberate their previously acid precipitable radioactive 5S RNA into the supernatant.

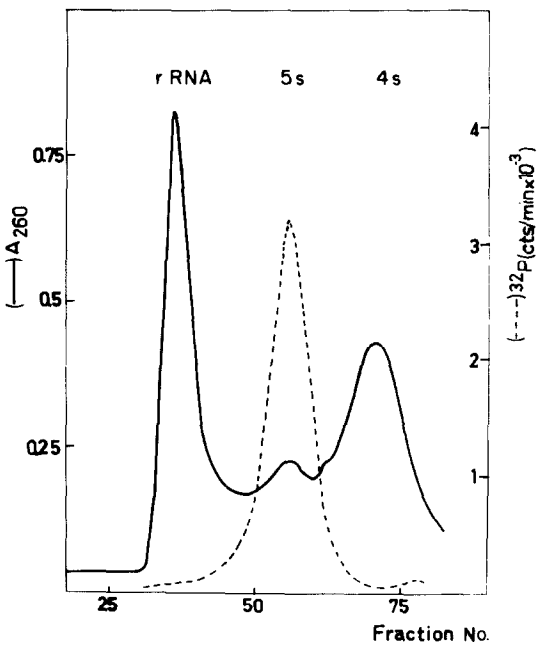

Figure 2. Sephadex gel filtration of RNA extracted from reconstituted ribosomal particles exposed to RNase T1.

LiCl stripped ribosomal particles, reconstituted in the presence of ^{32}P labeled 5S RNA and preincubated at 37° in the presence of mercaptoethanol, were sedimented, resuspended in KMT buffer and exposed to RNase T1 for 30 minutes at 37°, in a concentration of 5 µg RNase/100 µg total RNA. The particles were then sedimented and their RNA prepared by extraction with phenol at 4° in the presence of one mg/ml commercial sRNA, 0.3% sodium dodecyl sulfate and 1/6 volume macaloid. The RNA was precipitated by addition of 2 volumes of 95% ethanol, and 350 µg of the RNA was filtered at 4° through a column of Sephadex G100 (1.6 x 90 cm) equilibrated with 0.375M NaCl, 0.01M sodium acetate, pH 5.0, and 1% (v/v) methanol; the fraction size was 1.5 ml. The positions of ribosomal (rRNA), 5S RNA and 4S RNA are indicated.

- - - - ^{32}P (5S RNA)

———— A_{260} (ribosomal RNA of the particles and added carrier sRNA)

The radioactive 5S RNA contained in the RNase-treated particles which do sediment at 100,000 g was analysed, after phenol extraction of the particles, by Sephadex gel filtration (Figure 2) and MAK column chromatography (Figure 3). In the case of both T1 and pancreatic RNase treated particles, the recovered radioactive RNA coincides with the non-radioactive carrier 5S RNA, and no other peaks of radioactivity are seen in the region of lower molecular weight RNA. These findings suggest that the 5S RNA in the sedimented reconstituted particles was left intact by the RNase. On the contrary, the other ribosomal RNA's (16S and 23S RNA) of the sedimented particles were extensively degraded by the RNase, since, in Figure 3, there are no clearly distinguishable optical density peaks of 16S and 23S RNA, only a widebased slope of optical density material presumably representing a heterogeneous mixture of various sized RNA

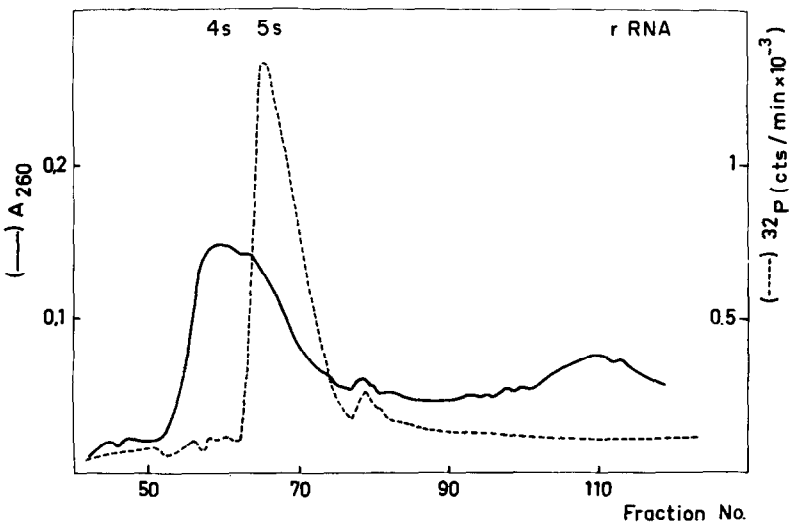

Figure 3. MAK column chromatography of RNA extracted from reconstituted ribosomal particles exposed to RNase T1.

The experimental conditions are the same as those indicated in Figure 2 except that the reconstituted particles were not preincubated in the presence of mercaptoethanol. MAK column chromatography of 350 µg of RNA was carried out as previously described (8); the fraction size was 3 ml. Elution positions of 4S RNA, 5S RNA and ribosomal (rRNA) are indicated.

- - - - ^{32}P (5S RNA)

———— A_{260} (ribosomal RNA of the particles and added carrier sRNA)

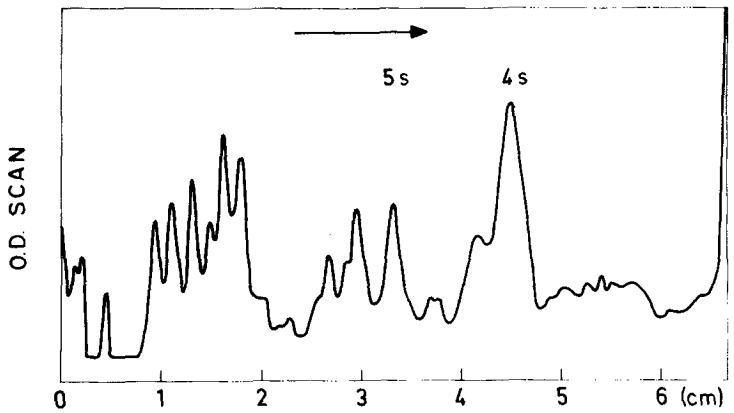

Figure 4. Acrylamide gel electrophoresis of RNA extracted from reconstituted ribosomal particles exposed to RNase T1.

A portion of the RNA sample, from the experiment shown in Figure 3, was fractionated by electrophoresis in gels of 10% acrylamide containing 7M urea. The gels were fixed and stained with acridine orange, then destained electrically and scanned for absorbance at 465 mμ using a Joyce Loebl Chromoscan with a blue filter. The tracing of such a scan is shown: the arrow indicates direction of migration of RNA during electrophoresis, and the numbers, the distance in cm from the origin; the positions of 4S RNA and 5S RNA, from the added carrier sRNA, are indicated.

breakdown fragments. The degradation of the high molecular weight ribosomal RNA's of the particles is confirmed by acrylamide gel electrophoresis, in the presence of 7M urea, of RNA extracted from RNase-treated particles (Figure 4): numerous bands of optically dense material are found between the top of the 10% gel, beyond which 16S RNA does not migrate, and the bands of 5S RNA and 4S RNA provided by carrier commercial soluble RNA added to the preparation. These supplementary bands must represent degradation products of the 16S RNA and 23S RNA of the particles.

The 5S RNA isolated from the RNase-treated particles after column chromatography (Figures 2 and 3) was completely digested with RNase T1 and oligonucleotide fingerprints of the digests were prepared. No qualitative or quantitative differences were noted in the oligonucleotides present in digests of this 5S RNA and those present in digests of control 5S RNA. In particular, the 5'-terminal and 3'-terminal oligonucleotides were present in normal molar yields.

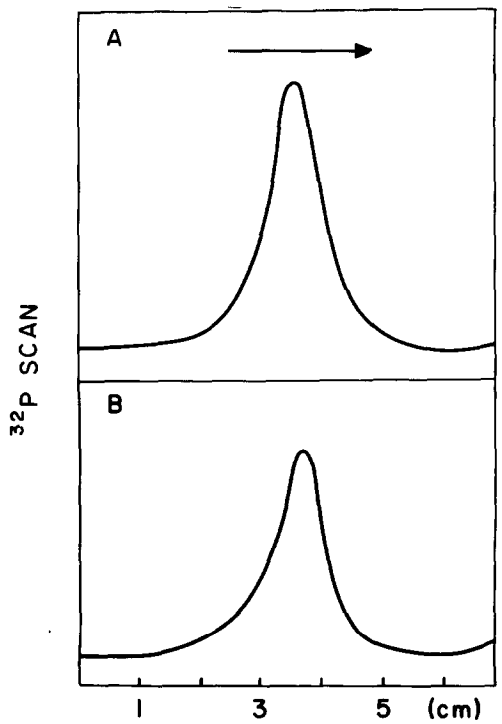

Figure 5. Acrylamide gel electrophoresis of normal 5S RNA and 5S RNA extracted from reconstituted ribosomal particles exposed to RNase T1.

^{32}P labeled 5S RNA initially added to the reconstitution mixture, and the RNA extracted from reconstituted ribosomal particles exposed to RNase T1 (experiment shown in Figure 2), were fractionated by electrophoresis in gels of 10% acrylamide containing 7M urea. The gels were sliced longitudinally and scanned for radioactivity using a Vanguard 880 Chromatogram Scanner. The arrow indicates the direction of migration of RNA during electrophoresis, and the numbers, the distance in cm from the origin.

A - ^{32}P scan of control 5S RNA

B - ^{32}P scan of RNA prepared from reconstituted ribosomal particles exposed to RNase T1.

Finally, the 5S RNA of RNase T1-treated particles was fractionated by acrylamide gel electrophoresis in the presence of 7M urea (Figure 5). The 5S RNA emerged as a single peak of radioactivity in the same position as control 5S RNA. It seems unlikely, then, that there are any "hidden nicks" in the primary structure of the 5S RNA of RNase T1-treated particles.

Experiments using pancreatic RNase yielded similar results as with

RNase T1-treated particles, with one exception. In one experiment, acrylamide gel electrophoresis (with 7M urea) of 5S RNA from pancreatic RNase treated particles yielded, in addition to intact 5S RNA, some lower molecular weight radioactive material. This latter finding must be reverified, and suggests the possibility of some "hidden nicks" in the 5S RNA of pancreatic RNase-treated particles.

DISCUSSION

The experiments described above reveal that 5S ribosomal RNA, attached to certain reconstituted ribosomal particles, is extremely resistant to degradation by RNase, under conditions where unattached 5S RNA is completely degraded. By all the criteria we tested, the 5S RNA remains completely intact at least in the case of T1 RNase-treated particles. These findings suggest that in reconstituted ribosomal particles, previously dissociated by LiCl, and presumably even more so in native 50S ribosomal subunits, the 5S RNA is situated deeply within the structure of the ribosome so that it is protected from the action of RNase. Therefore, it seems unlikely that 5S RNA plays any role at the surface of the ribosome such as acting as a bridge between 30S and 50S subunits, or helping to bind tRNA to the ribosome. A purely structural role for 5S RNA in the 50S ribosomal subunit is suggested.

ACKNOWLEDGEMENTS

We thank Professor R. Monier for many helpful discussions and for making facilities available for this work. Mrs. C. Doumeng and Miss R. Jourdan provided skilled technical assistance. This work was supported in part by grants 1-F3-GM-38,781 and 1-TO1-AM05581 of the USPHS and by grants of the CEA and DGRST (France). This work comprises part of doctoral thesis C.N.R.S. No. A.O. 2914, presented in 1970 by M. Reynier at the University of Marseille-Luminy.

REFERENCES

1. Marcot-Queiroz, J. and Monier, R., Bull. Soc. Chim. Biol. 49, 477 (1967).
2. Reynier, M. and Monier, R., Bull. Soc. Chim. Biol. 50, 1583 (1968).
3. Aubert, M., Monier, R., Reynier, M. and Scott, J.F., in Structure and Function of Transfer RNA, Proceedings of the Fourth FEBS Meetings, Vol. 3., Universitets Forlaget, Oslo, 1968.

4. Cannon, M., Krug, R., and Gilbert, W., J. Mol. Biol. 7, 360 (1963).
5. Forget, B. and Varricchio, F., J. Mol. Biol. (in press).
6. Hindley, J., J. Mol. Biol. 30, 125 (1967).
7. Aubert, M., Scott, J.F., Reynier, M., and Monier, R., Proc. Nat. Acad. Sci., 61, 292, (1968).
8. Marcot-Queiroz, J. and Monier, R., J. Mol. Biol. 14, 490 (1965).
9. Richards, E.G., Coll, J.A. and Gratzer, W.B., Anal. Biochem. 12, 452 (1965).
10. Sanger, F., Brownlee, G.G. and Barrell, B.G., J. Mol. Biol. 13, 373 (1965).
11. Forget, B.G. and Weissman, S.M., J. Biol. Chem. 243, 5709 (1968).