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10Sa RNA Is Associated with 70S Ribosome Particles in *Escherichia coli*[1]

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The intracellular distribution of 10Sa RNA in *Escherichia coli* was investigated in cell extracts. Northern hybridization revealed that a large fraction of 10Sa RNA cosediments with 70S ribosomes. When 70S ribosomes were dissociated into 50S and 30S subunits in the presence of low levels of Mg^{2+} ions, almost all of the 10Sa RNA disappeared from both subunits. The extent of the association of the 10Sa RNA with ribosomes was much enhanced during the growth phase of the cells. These results suggest the possibility that 10Sa RNA might function on the ribosomes in *E. coli* cells.

Key words: *Escherichia coli*, growth phase, 70S ribosome, S100 fraction, 10Sa RNA.

10Sa RNA is one of the small, stable RNAs found in *Escherichia coli* (*1, 2*). The *ssrA* gene for 10Sa RNA of *E. coli* is located at 56.5 min on the linkage map of the chromosome (*3*). Considerable amounts of this RNA (about 1,000 copies per haploid genome) are present in the cells (*4*). In our previous study (*5*), we showed that 10Sa RNA has an interesting structural feature: 7 nucleotides at the 5′ end and 28 nucleotides at the 3′ end of 10Sa RNA can be arranged in a structure that is equivalent to a half-molecule of tRNA (the acceptor stem and TΨC stem-loop; Fig. 1). 10Sa RNA has a G-U base pair in its stem that corresponds to the G-U pair, the determinant of the identity of $tRNA^{Ala}$ in the acceptor stem (*6, 7*), and it can be charged with alanine *in vitro*. Disruption of the *ssrA* gene that encodes 10Sa RNA causes a reduction in the rate of cell growth, in particular at 45°C, and a reduction in motility on semi-solid agar. Furthermore, a contribution of aminoacylation to the function of 10Sa RNA *in vivo* has been suggested, based on the results of mutational analysis of *ssrA*. In spite of the efforts invested in studies on the function(s) of 10Sa RNA (*8-10*), the precise function of this RNA remains to be determined.

To obtain further clues to the function of 10Sa RNA, we have investigated where and how 10Sa RNA can be found in *E. coli* cells. In this report, we describe our finding that 10Sa RNA is associated with 70S ribosomal particles.

MATERIALS AND METHODS

Fractionation of an E. coli Lysate and Purification of Ribosomes—*E. coli* W3110 cells, grown in LB broth to mid-logarithmic phase, were harvested and washed with ice-cold 0.2 M KCl. The cell pellet was suspended in buffer I [10 mM Tris-HCl (pH 7.6), 100 mM ammonium acetate, 10 mM magnesium acetate, 6 mM β-mercaptoethanol], and cells were disrupted by sonication over ice-water. The suspension was treated with DNase I (final concentration, 1 mg/ml) for 30 min at 4°C and centrifuged at $30,000 \times g$ for 20 min. Then the supernatant was further centrifuged at $100,000 \times g$ for 90 min and the supernatant and the pellet (resuspended in a small volume of buffer I) were used for assays as the "S100" fraction and the "crude ribosome" fraction, respectively. For further purification of ribosomes, the pellet after centrifugation at $100,000 \times g$ was washed three times in buffer II [20 mM Tris-HCl (pH 7.6), 1 M ammonium acetate, 10 mM magnesium acetate, 6 mM β-mercaptoethanol] and finally suspended in buffer I.

Sucrose Density Gradient Centrifugation—Between one and five A_{260} units of crude ribosomes were layered on a 10-30% sucrose gradient (4.95 ml) prepared in 10 mM Tris-HCl (pH 8.0), 30 mM KCl, and 10 mM magnesium acetate, and centrifuged in a Beckman SW50.1 rotor at 35,000 rpm for 150 min at 4°C. After the centrifugation, the solution was collected as 24 fractions through a hole in the bottom of the tube. To dissociate ribosome particles into subunits, the preparation of crude ribosomes was diluted 10-fold with Mg^{2+}-free buffer and fractionated by centrifugation under the same conditions as described above except that the concentration of magnesium acetate in the sucrose gradient solution was reduced to 1 mM.

Preparation of RNA and Northern Hybridization—RNA was isolated directly from samples (S100, crude ribosomes and each fraction from the sucrose density gradient after centrifugation) by successive extraction with phenol, phenol plus chloroform, and chloroform, with subsequent ethanol precipitation. When a low concentration of RNA was anticipated, unfractionated tRNA from *E. coli* was added (10 mg per tube) as a carrier during ethanol precipitation. For Northern hybridization, electrophoresis was carried out by the glyoxal method on a 1.4% agarose gel and RNA was blotted onto a nylon membrane (Biodyne; Pall BioSupport, East Hills, NY) by the capillary method.

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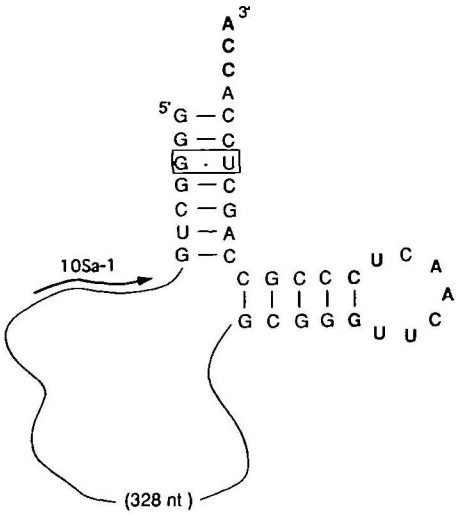

Fig. 1. **The tRNA-like structure of 10Sa RNA.** The total length of 10Sa RNA is 363 nucleotides. Bold letters indicate the conserved bases found in tRNAs. The G-U base pair that corresponds to the G-U pair, the determinant of the identity of $tRNA^{Ala}$, is boxed. The position of the synthetic probe 10SA-1 is shown by an arrow.

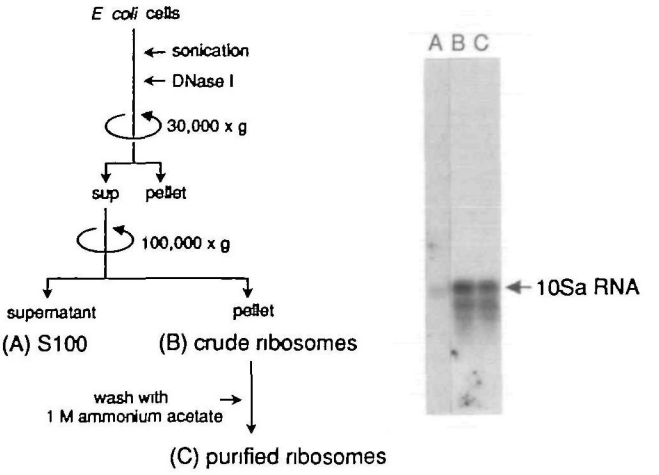

Fig 2. **Localization of 10Sa RNA.** (Left) The scheme for fractionation of an extract of *E coli* cells is shown (Right) RNAs extracted from fractions (A), (B), and (C) were examined by Northern hybridization The DNA fragment used as the probe was a 0 4-kb fragment from the *Acc*I site (in the promoter region of *ssrA*, which encodes 10Sa RNA) to a *Bsm*I site (artificially inserted just downstream of *ssrA*).

Hybridization was performed as described elsewhere (*11*). Probes were labeled with ^{32}P by use of the Multiprime DNA labeling system (Amersham Japan, Tokyo).

Preparation of Cross-Linked RNA—The preparation of crude ribosomes was diluted in buffer I to 5 A_{260} units/ml and irradiated with ultraviolet light on ice in Stratalinker (Stratagene Cloning Systems, La Jolla, CA). The dose of irradiation was 5×10^5 or 1×10^6 $\mu J/cm^2$. After irradiation, the sample was treated with proteinase K (final concentration, 0.2 mg/ml) at 37°C for 30 min and RNA was prepared by phenol extraction and ethanol precipitation.

Purification of 10Sa RNA—Five nanomoles of a synthetic oligonucleotide 10SA-1 [5′-TCGGCATGCACCTTGGGTTTCGCAA, custom-synthesized by Biologica (Nagoya); Fig. 1] was biotinylated at the 3′ end by use of Biotin-21-dUTP (Clontech Laboratories, Inc., Palo Alto, CA) and terminal deoxynucleotides transferase, and then the DNA was immobilized on 0.5 ml of streptavidin-conjugated agarose beads (ImmunoPure Immobilized Streptavidin; Pierce Chemical, Rockford, IL). The immobilized probe was allowed to hybridize with RNA extracted from 20 A_{260} units of ribosomes in 20 mM PIPES-NaOH (pH 6.4), 0.6 M NaCl, 0.2% SDS, 1 mM EDTA, 50% formamide, at 37°C. After a 16-h incubation, the preparation was washed twice with 2×SSC, 0.2% SDS; twice with 0.2×SSC, 0.2% SDS; and finally with H_2O at room temperature. Hybridized RNA was then eluted in a small volume of H_2O by incubation at 95°C for 2 min.

RESULTS

Localization of 10Sa RNA—To determine where and how 10Sa RNA exists in *E. coli* cells, we fractionated a cell lysate by two successive centrifugations. Northern hybridization with a probe for 10Sa RNA was carried out with the RNA extracted from each fraction. Most of the 10Sa RNA was detected in the pellet after centrifugation at 100,000× *g* (Fig. 2). This fraction contained ribosomes for the most part and, thus, we refer to it as the crude ribosome fraction. We further purified this fraction by the method used for purification of ribosomes, with washing in high-salt buffer. After three washes, 10Sa RNA was still associated with the ribosome fraction (Fig. 2, lane C).

The crude ribosomes were fractionated by centrifugation on a sucrose density gradient and the localization of 10Sa RNA in the various fractions was examined by Northern hybridization. 10Sa RNA was dispersed all over the gradient, with a significant peak in the fractions in which 70S ribosome was found (Fig. 3A). When conditions for the centrifugation (sucrose concentration, centrifugation time, and so on) were altered, the peaks of 10Sa RNA and 70S ribosome were again found at the same position (data not shown). Ribosomes are interconvertible *in vitro* between 70S monomers and 50S and 30S subunits, depending on the concentration of Mg^{2+} ions and monovalent cations. We fractionated the crude ribosomes on a sucrose density gradient in the presence of 1 mM Mg^{2+} ions (Fig. 3B). The amount of 70S particles decreased and the amounts of 50S and 30S subunits increased, as compared with the results in the presence of 10 mM Mg^{2+}. However, the proportion of the total 10Sa RNA found in the 50S and 30S fractions did not increase. Some of the 10Sa RNA remained in the same fraction as the 70S ribosomes, and the rest of the 10Sa RNA was found near the top of the gradient. These results suggest the possibility that 10Sa RNA coexists with the 70S ribosome. However, we cannot rule out the possibility that 10Sa RNA is a part of an unidentified complex that has the same sedimentation coefficient as 70S ribosomes.

10Sa RNA Is Associated with 70S Ribosome Particles—To confirm that 10Sa RNA associates with 70S ribosomes, we carried out an RNA cross-linking experiment. RNA was extracted from crude ribosomes that had been irradiated by ultraviolet light. In this preparation of RNA, we detected two extra bands in addition to the band of 10Sa RNA monomer by Northern hybridization with the probe for 10Sa RNA, although the upper band was only detected in

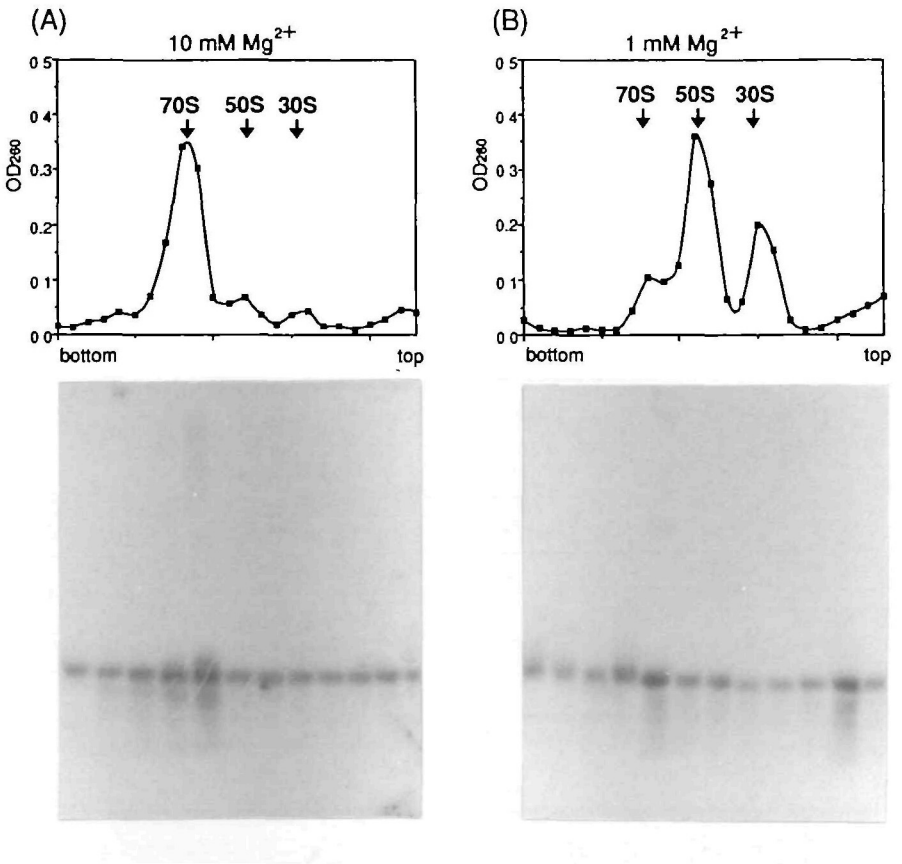

Fig. 3 **10Sa RNA is co-localized with 70S ribosome particles in cell extracts.** Crude ribosomes were fractionated by sucrose density gradient centrifugation at high (A) and low (B) concentration of Mg^{2+} ions. The absorbance at 260 nm of each fraction is shown in the upper panels. RNA extracted from parts of the fractions was combined and analyzed by Northern hybridization, as in Fig. 2, and the results are shown in the lower panels.

the over-exposed autoradiograms (Fig. 4A). The intense band had a slightly lower mobility than that of 16S rRNA, and the other band had a similar mobility to that of 23S rRNA. The intensity of these extra bands depended on the dose of ultraviolet light, suggesting that they represented cross-linked products. Since the large amounts of free 16S and 23S rRNA molecules in this preparation interfered with the identification of these cross-linked products, we purified 10Sa RNA and the RNA that was covalently linked to 10Sa RNA from the mixture of RNAs, using a probe that had been immobilized on a solid support. The purified RNA was then used for Northern hybridization (Fig. 4B). The probe for 10Sa RNA detected 10Sa RNA monomer and the two cross-linked products. The probe for 16S and 23S rRNA hybridized to RNA molecules that had exact the same mobility as the two cross-linked products, the more abundant and the less abundant product, respectively. These hybridization signals could be seen only in the case of the ultraviolet-irradiated sample. The results indicated that the two products were 10Sa RNA molecules cross-linked with 16S rRNA and 23S rRNA, respectively. It is thus apparent that 10Sa RNA is associated with 70S ribosome particles in such a way that one part of 10Sa RNA makes contact with the 30S subunit and another part makes contact with the 50S subunit. This observation is consistent with the distribution of 10Sa RNA when ribosomes are fractionated under low-Mg^{2+} conditions (Fig. 3B), namely, 10Sa RNA was not selectively associated with either subunit and 10Sa RNA was separated from ribosomes when 70S ribosomes were dissociated into the two subunits. It seems that 10Sa RNA is associated more with 16S RNA than with 23S RNA from the cross-linking experiments.

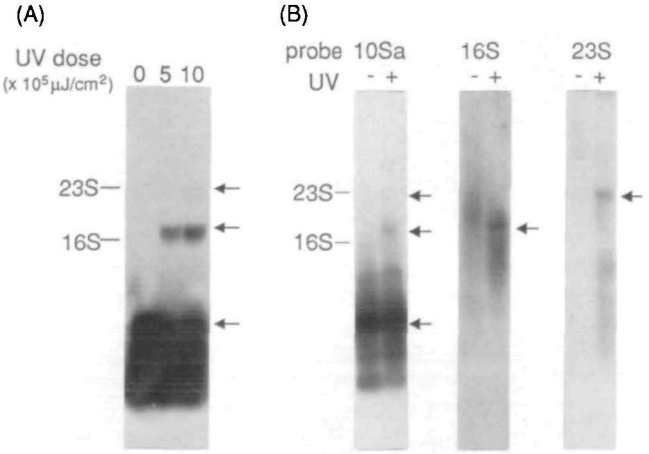

Fig. 4. **Cross-linking of 10Sa RNA and rRNAs by ultraviolet light.** (A) RNA was extracted from ribosomes that had been irradiated by ultraviolet light, and used for Northern hybridization. The probe was the same as that used for the hybridizations in Figs. 2 and 3. (B) RNA that hybridized to the 10SA-1 probe (Fig. 1) was purified from the cross-linked mixture of RNA in (A) and examined by Northern hybridization. The fragments used as probes were as follows. for 10Sa RNA, the same one as that used in the hybridizations in Figs 2, 3, and 4(A), for 16S, a 0.7-kb fragment from the *Eco*RI site to the *Sma*I site of *rrsH* (encoding 16S rRNA); for 23S, a 0.8-kb fragment from the *Sph*I site to the *Sal*I site of *rrlH* (encoding 23S rRNA). Arrows in (A) and (B) indicate the positions of the 10Sa RNA monomer (lowest arrows) and the two cross-linked products.

However, we cannot yet conclude whether or not this difference is essential for the association between 10Sa

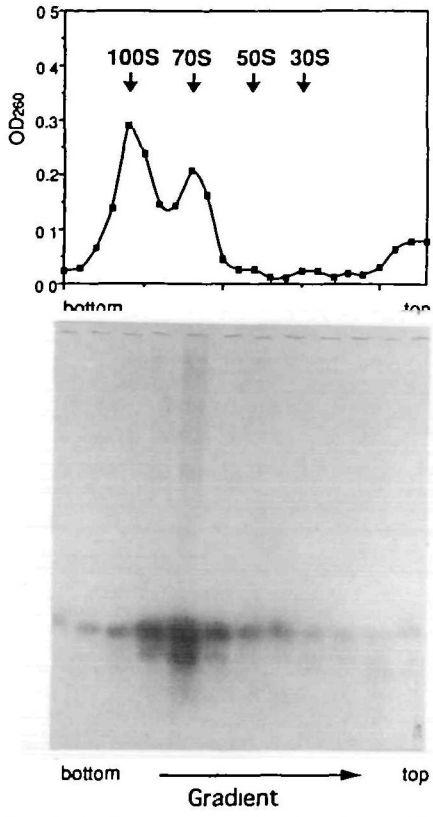

Fig. 5. **10Sa RNA is not associated with 100S ribosome dimers.** Ribosomes were prepared from a stationary-phase culture (incubated for 24 h) and assayed as described in the legend to Fig. 2 (centrifugation was carried out in the presence of 10 mM Mg^{2+} ions).

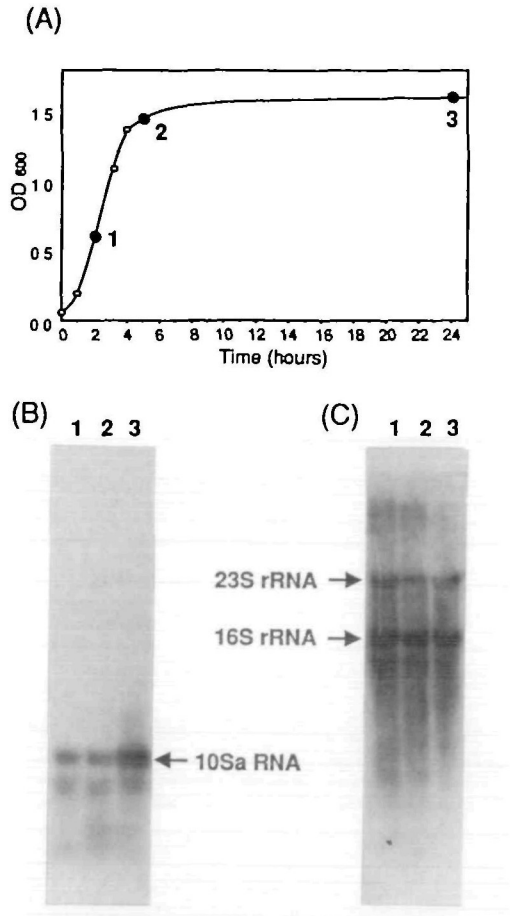

Fig. 6 **The amount of 10Sa RNA at different stages of growth.** (A) Growth curve of the culture Numbers 1, 2, and 3 show the times at which cells were harvested and ribosomes were prepared. (B) and (C) Ribosomes prepared from the culture at three stages (1, 2, and 3) of growth were fractionated by sucrose density gradient centrifugation and RNA was extracted from the 70S fractions. The same amount of RNA was subjected to electrophoresis in each lane, and Northern hybridization was carried out with the probes for 10Sa RNA (B) or for 16S and 23S rRNA (C). The amount of RNA per lane and the duration of exposure to X-ray film in (B) were about 20 and 5% of those in (C), respectively.

RNA and ribosomes.

The Amount of 10Sa RNA Is Related to the Growth Phase—To elucidate the physiological significance of the association of 10Sa RNA with ribosomes, we analyzed ribosomes prepared at different phases of the growth cycle. At the stationary phase, 70S ribosomes undergo dimerization to form 100S particles, which are considered to be a storage form of ribosomes (*12*). We prepared ribosomes from a stationary-phase culture of W3110 and examined the 100S fraction for the presence of 10Sa RNA. As shown in Fig. 5, 10Sa RNA was present mainly in the 70S fraction, and little 10Sa RNA was detected in the 100S fraction. However, the amount of 10Sa RNA detected in the 70S fraction seemed to be larger in the stationary-phase sample than in the logarithmic-phase sample. In the experiment for which the results are shown in Fig. 6, RNA was extracted only from the 70S fractions at three stages of cell growth, and equal amounts of RNA were subjected to electrophoresis in each lane. At the transition from the logarithmic phase to the stationary phase, the level of 10Sa RNA did not increase much (Fig. 6B, lane 2). However, in the stationary phase (cells cultured for 24 h) the relative level of 10Sa RNA was clearly greater (lane 3) than that in the logarithmic phase. As estimated from the intensity of the hybridization signals, the number of 10Sa RNA molecules was about 0.5 and 2% of that of 16S rRNA molecules in the logarithmic phase and stationary phase, respectively.

DISCUSSION

10Sa RNA has been found not only in *E. coli* but also in other microorganisms, namely, *Alcaligenes eutrophus* (*13*), *Mycobacterium tuberculosis* (*14*), *Mycoplasma capricolum*, and *Bacillus subtilis* (*15, 16*). Moreover, Ushida and Muto showed that a large part of the 10Sa RNA in *B. subtilis* and *M. capricolum* cells cosediments with 70S ribosomes at high concentrations of Mg^{2+} ions, while at low concentrations of Mg^{2+} ions, which cause 70S ribosomes to dissociate into 50S and 30S subunits, the RNA appeared solely in the soluble fraction (*15*). In this study, we obtained the same result for the 10Sa RNA of *E. coli*. To confirm this, it will be necessary to demonstrate the association between 10Sa RNA and ribosomes by means of *in vitro* reconstruction experiments.

Recently, we isolated a novel temperature-sensitive mutant with a deletion within the *ssrA* gene for 10Sa RNA.

The temperature-sensitive (ts) phenotype of this mutant could be overcome by introduction of the wild-type *ssrA* gene but not by mutant forms of *ssrA* (*17*). The ts mutation was mapped within the *prs* gene, which encodes phosphoribosyl pyrophosphate synthetase. The existence of this type of mutant strongly suggests that 10Sa RNA can associate with phosphoribosyl pyrophosphate synthetase in a functional manner. In addition, from the results presented herein, we speculate that 10Sa RNA might be associated with the ribosome in a resting form or in a post-release form, rather than in an active (translating) form, because the amount of 10Sa RNA was increased in the stationary phase. Furthermore, it should be noted that ribosomes associated with 10Sa RNA are a small fraction of the total ribosomes, even if all the 10Sa RNA in the cell can associate with them. Thus, 10Sa RNA might not simply be involved in translation, but might act on ribosome particles post-translationally by some unknown mechanism. At present, however, we have no evidence to support this possibility and further biochemical and genetic studies of the associations between 10Sa RNA, phosphoribosyl pyrophosphate synthetase and ribosome particles are now required.

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