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Small Stable RNAs from *Escherichia coli*: Evidence for the Existence of New Molecules and for a New Ribonucleoprotein Particle Containing 6S RNA

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Small stable RNA molecules of *Escherichia coli* other than 5S (rRNA) and 4S (tRNA) were studied. Two of the molecules corresponded to 4.5S and 6S RNA, which have been reported previously. The third stable RNA molecule, 10S RNA, has not been described before. RNA labeled with $^{32}P_i$ or [^{14}C]uracil for a relatively long time, when separated in 5%/12% tandem polyacrylamide gels, displayed three bands corresponding to 10S, 6S, and 4.5S RNA in addition to rRNA and tRNA bands. These RNAs were stable in pulse-chase-labeling experiments. The amount of these RNAs was small, comprising only 0.2 to 0.5% of the total ^{32}P incorporation. However, this amount represented a large number of molecules; for 6S and 4.5S, it was about 1,000/DNA molecule. These three RNAs were found in the postribosomal supernatant fraction. None of them was found in purified nucleoid fractions in which the tightly coiled DNA molecules were contained. Of these three RNAs, 6S RNA was unique in that it seemed to exist in a ribonucleoprotein particle. All these RNAs, as well as tRNA, were very stable in the cell under various physiological conditions. 5S RNA was less stable. On the other hand, purified 6S RNA was more susceptible than tRNA to cell nucleases when incubated with cell extracts, suggesting that, being in a particle, it is protected from cell nucleases.

An early report on 6S and 4.5S RNA was first made by Hindley when he detected two novel RNA molecules in a crude preparation of tRNA (11). The fingerprinting analysis suggested that they were distinct entities. Later, Goldstein and Harwood confirmed that 6S RNA is a normal component in *Escherichia coli* cells (7), and Griffin identified 4.5S RNA in several strains of *E. coli* (8). The nucleotide sequences of 6S RNA and 4.5S RNA were determined (4, 9), and the precursor molecules of 6S and 4.5S RNA have been recently identified (3, 10). The 6S and 4.5S RNAs were shown to be exclusively nonribosomal (7, 9). Under conditions of amino acid starvation in a stringent stain, the synthesis of 4.5S RNA was under stringent control, as was the synthesis of 5S rRNA and tRNA; however, the synthesis of 6S RNA was not under stringent control (13). Here we shall further characterize these small stable RNA molecules as well as introduce a new RNA component which we refer to as 10S RNA.

MATERIALS AND METHODS

Materials. Carrier-free $^{32}P_i$ (6 to 8 mCi/ml) was purchased from Amersham/Searle, and 2-[^{14}C]uracil (59 mCi/mmol) was purchased from New England Nuclear Corp. Acrylamide and *N,N'*-methylene-bisacrylamide were obtained from Eastman and were recrystallized with ethylacetate and acetone, respectively, before use. Sodium dodecyl sulfate (SDS) was from Fisher Scientific Company. Kodak X-ray film (NS 2T, 12.3 by 17.5 cm) was used for autoradiography.

Growth and labeling of cells. *E. coli* strain D10 requiring methionine was used throughout these studies. For long-term labeling with $^{32}P_i$, cells were grown at 37°C in low-phosphate medium (15) supplemented with $^{32}P_i$ (2 to 5 μCi/ml) for 6 to 7 h until the culture attained an absorbancy at 560 nm of 1.0 to 1.2. For short-term pulse labeling, cells were grown to an absorbancy at 560 nm of 0.2 to 0.3, and 10 μCi/ml of $^{32}P_i$ was added for 20 min. For the preparation of [^{14}C]uracil-labeled cells, cells were grown as above in minimal medium (1), and [^{14}C]uracil (5 to 10 μCi/ml) was added.

Preparation of RNA samples for electrophoresis. To prepare ^{32}P or [^{14}C]uracil-labeled RNA for electrophoresis, we essentially followed the rapid-lysis procedure described in reference 6. The lysis buffer in our system was made of 0.6% SDS, 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.5, 10 mM Na_2 ethylenediaminetetraacetic acid, 20% sucrose, and 0.02% bromophenol blue.

Analysis of lysates on sucrose density gradients. Samples were prepared by sonic treatment. Ten milliliters of a long-term ^{32}P-labeled culture was chilled quickly by adding ice. The cells were centrifuged and washed twice with TM2 buffer (0.01 M

Tris-hydrochloride [pH 7.5]-0.01 M magnesium acetate-1 mM dithioerythritol). The final pellet was resuspended in 0.2 ml of the same buffer and sonically treated three times for 30-s intervals with a Bronwill Biosonik sonic oscillator. The clear lysate was centrifuged at 20,000 × g for 10 min, and the supernatant was layered on the gradient.

In some experiments, the lysate after sonic treatment was further extracted with phenol as described in reference 12. In some cases, samples for the sucrose gradients were centrifuged, and the cells were resuspended in 1 ml of hot (95°C) SDS solution (1% SDS-200 mM sodium chloride-40 mM Na_2 ethylenediaminetetraacetic acid-20 mM Tris-hydrochloride, pH 7.5).

Lysates treated with protease (repurified protease from Sigma Chemical Co.), were prepared by sonic treatment as described above. The lysate was incubated with 500 μg of protease per ml for 1 h at 37°C in 0.01 M Tris-hydrochloride (pH 7.5)-0.01 M magnesium acetate-0.1 M sodium chloride. To remove residual ribonuclease (RNase) from the protease prior to digestion, it was necessary to self-incubate it in a similar buffer for 5 h at 37°C. After this treatment, the protease kept about 90% of its potency, as judged by its action on a crude extract of *E. coli* which was labeled with [^{3}H]leucine. The ribonuclease activity of the protease was tested for endo- or exonucleolytic cleavages of ^{32}P-labeled 23S rRNA by analysis of reaction mixtures incubated for 60 min at 37°C on thin-slab polyacrylamide gels (6, 15).

Samples were layered on top of a 4-ml 10 to 30% linear sucrose density gradient in TM2. The gradient was centrifuged at 300,000 × g for 4 h in SB-405 IEC rotor. Fractions of 0.2 to 0.25 ml were collected, and portions of each fraction were analyzed on 5%/12% tandem polyacrylamide gels to display their RNA content.

Polyacrylamide gel electrophoresis. Electrophoresis of cell lysates was carried out in a slab gel system (10 by 14.5 by 0.15 cm) (6, 15, 16). Large RNAs were analyzed on 3%/12% tandem polyacrylamide gels as detailed in reference 6. The gel had a 4-cm retaining layer of 12% polyacrylamide covered with 6 cm of 3% gel. In such a gel, DNA remains at the origin, 23S and 16S RNA are resolved into two distinct bands, and small RNAs are displayed in the 12% layer (see also reference 6). (We compared such gels to 1.5% polyacrylamide-0.5% agarose gels [5] and found that these assumptions were justified.) Small RNAs were separated on 5%/8% or 5%/12% tandem polyacrylamide gels as described in references 6, 15, and 16. The running buffer in our system was Tris-glycine buffer (15) supplemented with 0.1% SDS. Approximately 20,000 cpm were loaded in each slot. Gels were run at 100 V for the first 20 min and then at 150 V for 2 to 3 h or until the bromophenol blue marker reached 1 cm from the end of the gel. Subsequently, gels were dried and placed on X-ray films overnight for autoradiography. In these gels, DNA, 23S RNA, and 16S RNA were retained in the 5% gel, and the smaller RNAs entered the higher concentration of acrylamide and were separated in it.

Estimation of the number of RNA molecules in the cell. The number of RNA molecules per DNA molecule was estimated. Long-term $^{32}P_i$-labeled cells grown in low-phosphate medium were opened by the rapid lysis method (6) and separated in both 3%/12% and 5%/12% gels. Quantitation of gels was done as detailed in references 6 and 15. By quantitating the bands in 3%/12% gels, the ratio of DNA, 23S RNA, and 16S RNA was determined. This ratio was used to calculate the quantities of the large molecules trapped in the 5% layer of 5%/12% gels, and thus the percentage of the DNA and various RNA molecules could be calculated. It follows that the number of RNA molecules per DNA = (molecular weight of DNA per molecular weight of RNA) × (counts per minute of RNA per counts per minute of DNA). It has long been known that the amount of rRNA in the cell depends on the rate of growth (19). The rate of growth in our system was measured to be about one doubling per h. The number of rRNAs per DNA molecule was calculated by this method, and the result was compared to the known value at the corresponding growth rate. We found a close agreement between the different methods (see Results).

Preparation of nucleoids. Nucleoids were prepared as described by Pettijohn and Hecht (17). Long-term ^{32}P-labeled cells were centrifuged and resuspended in a solution of 10 mM Tris-hydrochloride, pH 8.1-10 mM sodium azide-100 mM sodium chloride. Lysozyme (4 mg/ml) was added, and the mixture was kept on ice for 2 to 3 min. An equal volume of the solution containing 1% Brij 58, 2 M sodium chloride, 10 mM Na_2 ethylenediaminetetraacetic acid and 0.4% deoxycholate was added, and the mixture was shaken at 20°C until it became clear. The clear lysate was centrifuged at 4,000 × g for 5 min, and the supernatant was centrifuged on a 10 to 30% linear sucrose density gradient for 25 min at 25,000 × g in SB-405 rotor. Fractions were analyzed in a 3%/12% tandem polyacrylamide gel, and those fractions containing DNA were pooled as nucleoid fractions.

Elution of RNA from gels and test for stability of RNA in vitro. The method for elution of RNA from the gel was based on that described by Ikemura and Dahlberg (12). Stability of RNAs in vitro was determined by digestion of purified RNAs with crude cell extract. Frozen cells of *E. coli* MRE600, which is devoid of RNase I, were grown with alumina (20) and extracted with TMK buffer (0.01 M Tris-hydrochloride [pH 7.5]-0.05 M potassium chloride-4 mM magnesium acetate). The supernatant after centrifugation at 12,000 × g for 15 min was used as crude cell extract. RNAs eluted from gels and precipitated with ethanol were resuspended in TMK buffer, the crude extract preparation was added to a final protein concentration of 15 mg/ml, and the digestion was carried out at 30°C for up to 2 h. Samples were analyzed by electrophoresis in a polyacrylamide gel, and RNA bands were quantitated to measure their stability.

RESULTS

Some features of the three small stable RNAs. As shown in Fig. 1 (left), when long-term $^{32}P_i$- or [^{14}C]uracil-labeled cells were lysed, and their RNA were separated in a 5%/12% tandem polyacrylamide gel, only a few distinct

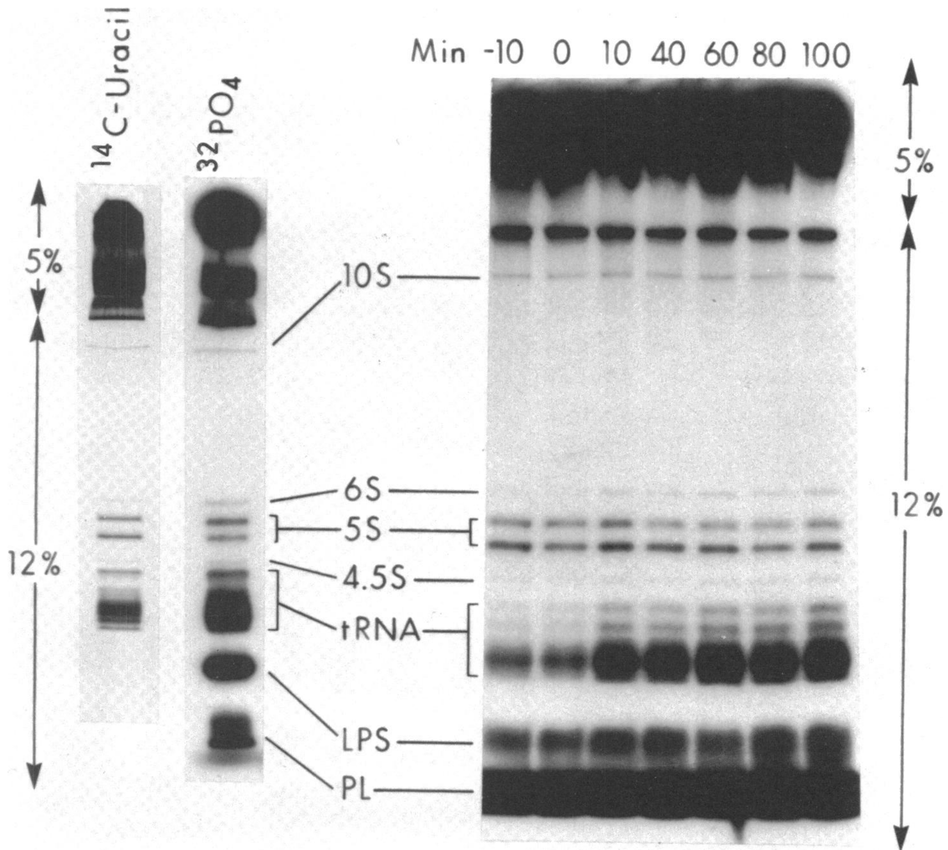

FIG. 1. *Stable RNA species of E. coli. The two left lanes show [^{14}C]uracil and long-term (7 h) ^{32}P-labeled cell lysates separated on a tandem 5%/12% gel. 10S, 6S, 5S, 4.5S, and tRNA bands appear in both lanes. Lipopolysaccharides (LPS) and phospholipids (PL) are labeled with ^{32}P only (2). Seven right lanes represent samples collected during phosphate starvation in a TM medium containing peptone (6) separated in a 5%/12% gel. Cells were labeled with $^{32}P_i$ for 2 h prior to starvation. Time 0 indicates the beginning of starvation. The 6S band became darker as cells were starved for phosphate due to the relative stability of 6S compared with rRNA (see text).*

bands could be seen. All of the bands which appear in the 12% part of the gel in the first lane represent RNA molecules, for they are labeled with [^{14}C]uracil as well as with $^{32}P_i$ and are sensitive to pancreatic RNase. In Fig. 1, the bands corresponding to 4.5S and 6S RNA have been previously described (3, 4, 7-11), whereas the band at the top of the 12% part of the gel represents a thus far unknown band of RNA which we call 10S RNA. (This is a first approximation; precise S-value measurements were not carried out.) When cells were labeled for a short time (20 min) with $^{32}P_i$ and then rifampin was added to block further RNA synthesis, with time, many of the RNA bands including the 10S RNA disappeared while the 4.5S, 6S, and tRNA remained stable (Fig. 2). These results suggested that 10S RNA represents an unusual molecule in that it is stable under normal growth conditions, but unstable when RNA synthesis is blocked. This was confirmed in further experiments. Further analysis in 5%/8% gels revealed that the 10S band contained at least two molecules, both of which were always stable when analyzed by pulse-chase experiments but could have different stabilities when measured after RNA synthesis was blocked by rifampin. As can be seen in Fig. 3, 6S RNA shows no change in stability in a pulse-chase labeling or in a rifampin-decay experiment, while the RNA molecules in the 10S band are stable in a pulse-chase experiment, but only one of these molecules remained stable after RNA synthesis was blocked by rifampin. Further experiments

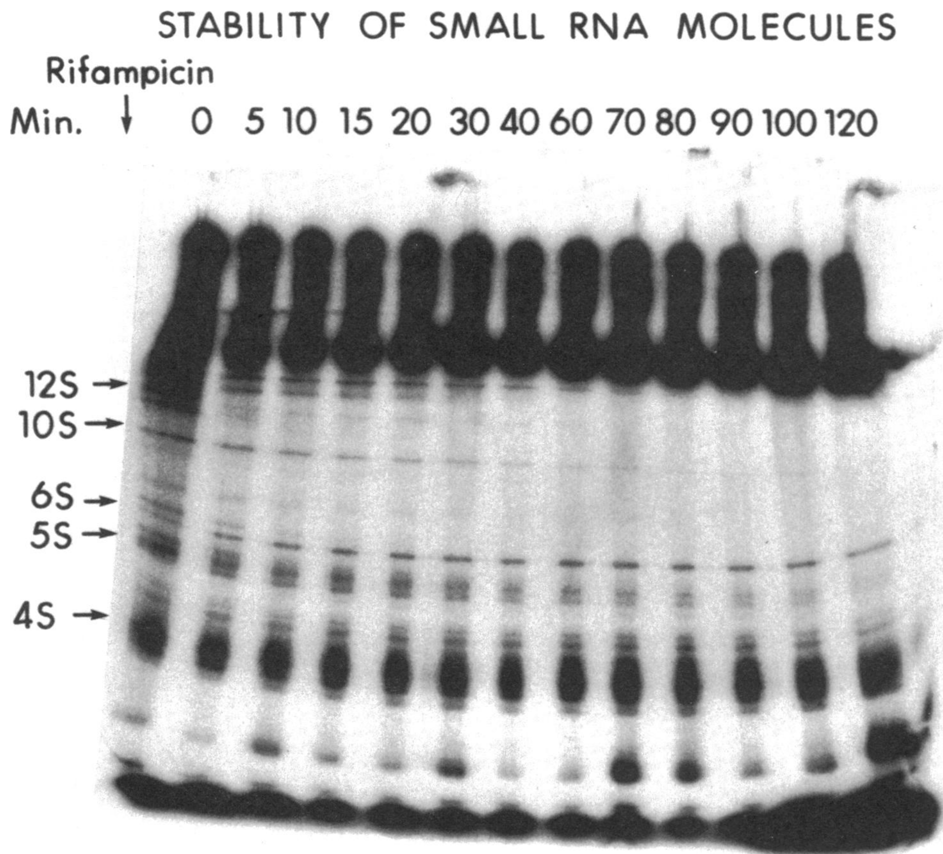

FIG. 2. *Stability of RNA molecules when RNA synthesis is blocked by rifampin. Short-term (20 min) $^{32}P_i$-labeled cells were sampled at various times after the addition of rifampin and run in a 5%/12% polyacrylamide gel.*

showed that after long-term labeling, two similar RNA molecules, as evident from fingerprinting analysis, each about 500 nucleotides long, can be isolated from the 10S region of the gel (B. Ray, unpublished observations).

We found that 6S RNA was stable during various starvations, such as starvation for carbon or nitrogen sources. During phosphate starvation (Fig. 1, right) the level of 6S RNA seemed to increase; however, detailed measurements of the various macromolecules during continuous labeling and short time pulses showed that this increase resulted from the relative stability of 6S RNA as compared with rRNA and from the fact that during phosphate starvation the newly synthesized RNA came from preexisting labeled RNA which was degraded.

Although 6S RNA was extremely stable in vivo, it was not as stable in vitro. 6S, 5S, and 4S RNA were purified from a gel and incubated with crude cell extract to compare the stability of the purified RNAs in vitro. The results showed that, while 6S RNA was the most stable of the three in vivo, in vitro it was less stable than 4S RNA. After 2 h of incubation, only 15% of the 4S RNA was degraded, whereas about 60% of 6S and 5S RNA were degraded. In all cases, control samples without enzyme showed no appreciable degradation at the end of the incubation period.

The amount of the small stable RNAs is low compared with rRNAs or tRNAs. 10S RNA comprised only about 0.5% of the total ^{32}P material, and 6S RNA and 4.5S RNA constituted only about 0.2% each, but these amounts represented a large number of molecules per cell. (For further information regarding the relative quantities of all the phosphate-containing macromolecules in *E. coli*, see reference 2, Table 2.) The number of molecules was estimated for 6S and 4.5S RNA as described in Materials and Methods. We estimated that there were approx-

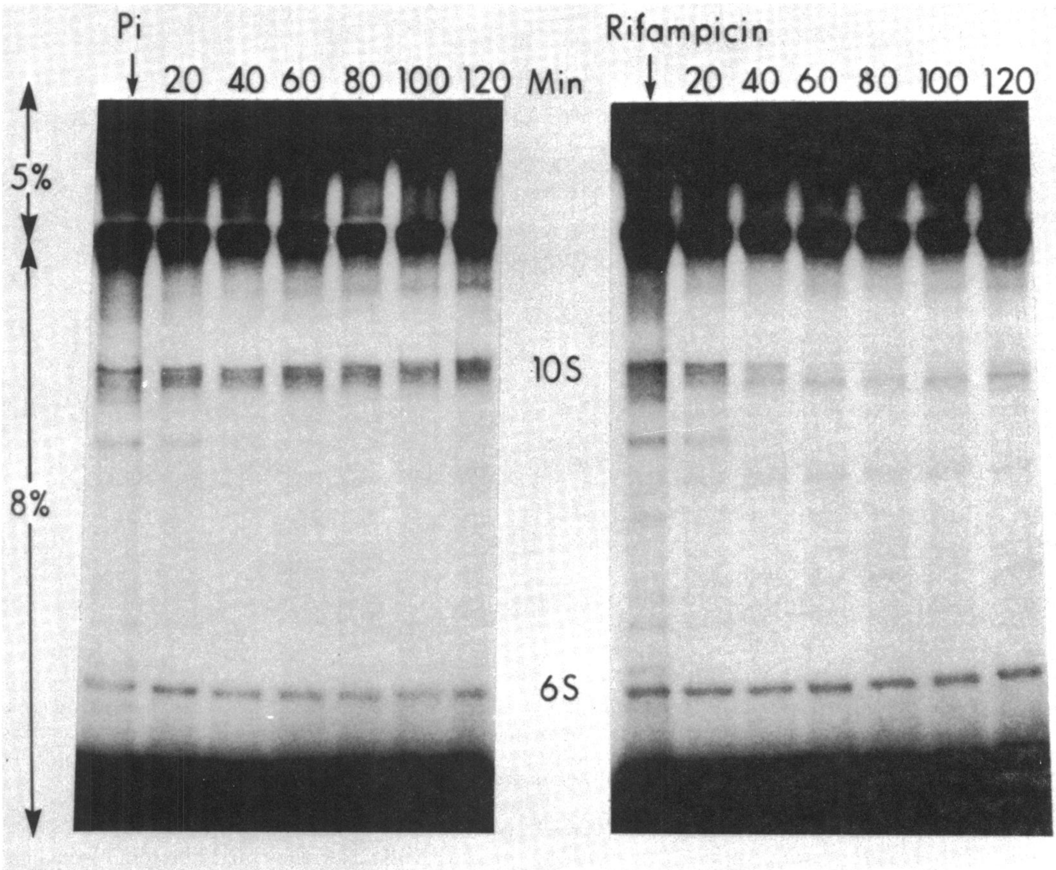

FIG. 3. *Comparison of the stability of 10S RNA in a pulse-chase and in a rifampin-decay experiment. Cells were short-term labeled with $^{32}P_i$, and to one-half, unlabeled PO_4^{3-} was added to make a 0.1% final concentration, whereas to the other half, 400 μg of rifampin per ml was added. Samples were taken every 20 min and analyzed in a 5%/8% gel. Part of the bottom of the gel is not shown. The separation of the faster-migrating components on such a gel was not adequate.*

imately 1,000 to 1,500 molecules of 6S RNA and about 1,000 to 1,400 molecules of 4.5S RNA per DNA molecule. Under the same growth conditions, the number of ribosomes was calculated to be about 5,000. The number of molecules of each of the two components in the 10S band was estimated to be about 500/DNA molecule. This estimate is less certain, since the 10S band contains more than a single species, and the molecular weight estimation for the top part of the 12% gel, without proper markers, is not completely reliable.

Cellular localization. Our results confirm the finding that 6S and 4.5S RNA are exclusively nonribosomal (7, 9). The molecules were found in the supernatant fraction after centrifugation of the ribosomes at 300,000 × *g*. In addition to the 6S and 4.5S RNA, 10S RNA was also nonribosomal, being present in the supernatant after the ribosomes had been removed. Such experiments were carried out in the absence of sucrose (to prevent possible dissociation of RNA from ribosomes), and extracts were prepared in 0.1 or 10 mM Mg^{2+}.

Since studies with the nucleoid of *E. coli* suggested that its compactness is maintained by RNA (17), we wanted to find out whether the nucleoid was enriched for any of these species of RNA. Therefore, a preparation of nucleoids was made and sedimented in a 10 to 30% sucrose gradient for 25 min at 25,000 × *g* (see Materials and Methods). Portions of fractions were analyzed in 3%/12% gels (Fig. 4), and fractions containing the nucleoids as well as some other fractions were subsequently analyzed in 5%/12% gels to reveal their RNA content. Prior to the second electrophoresis, SDS was added to the fractions (to a final concentration of 0.1%) and incubated

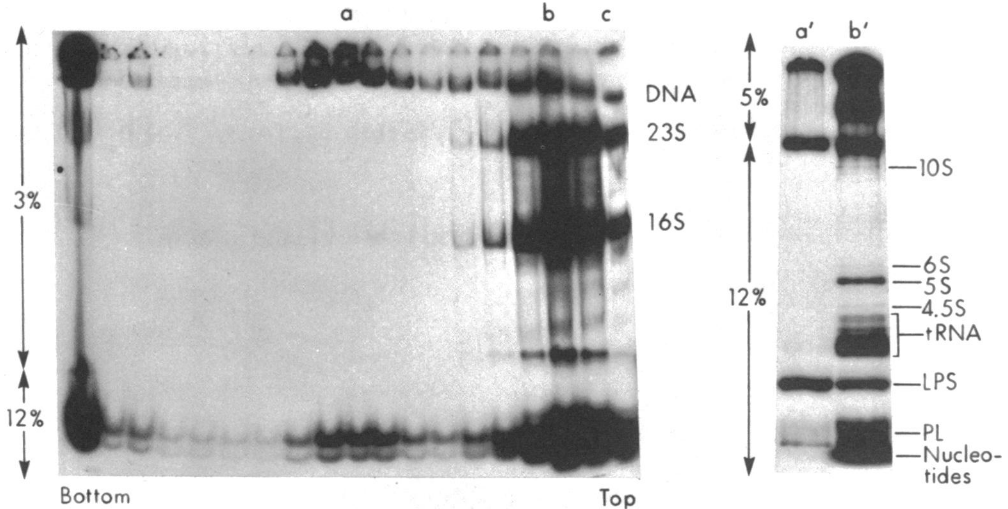

FIG. 4. *Test for enrichment of 10S, 6S, or 4.5S RNA in the nucleoid. The figure is the composite of two different gels. On the left, fractions from a gradient of a* ^{32}*P-labeled crude nucleoid preparation are displayed. A sample of the material prior to the fractionation is displayed in lane c. Fraction a, which contains much of the DNA (nucleoid fraction), and fraction b, which is enriched for rRNA, were treated with SDS as discussed in the text and analyzed in a 5%/12% gel as shown on the right (a', b').*

at room temperature for 30 min or at 90°C for 3 min. Either method was sufficient to release the RNA. As shown in Fig. 4, none of the three RNAs were present in the nucleoid fractions. All three RNAs remained in the slow-sedimenting region of the gradient above the nucleoids.

6S RNA is in a particle. Long-term ^{32}P-labeled cells were sonically treated and fractionated in a 10 to 30% linear sucrose gradient in TM2 buffer. Portions of each fraction were analyzed in a 5%/12% tandem polyacrylamide gel. The autoradiogram of the gel is shown in Fig. 5. Surprisingly, 6S RNA appears in an unexpected position in the gradient. It sedimented faster than 10S RNA. In the experiment shown in Fig. 5, extracts were prepared in 0.1 mM Mg^{2+}. Similar results were obtained when extracts were prepared in 10 mM Mg^{2+}.

When cell lysates prepared as above were extracted with phenol, and the resulting total RNA, free of proteins, was similarly analyzed by centrifugation in a sucrose gradient, the position of 6S RNA in the gradient moved from its former position to a new position between 10S and 4.5S RNA. A similar change of position occurred when cells were extracted with hot SDS buffer (see Materials and Methods) and separated in gradients. If proteins were associated with 6S RNA, they might be removed when the cell lysate was digested with RNase-free protease. Sucrose gradient analysis of such a digest is shown in Fig. 6. The result shows that the sedimentation of the 6S RNA band in the gradient was slower than the 10S RNA band, as in phenol- or SDS-extracted samples. On the other hand, the migration in the sucrose gradients of 10S and 4.5S RNA was not affected by the protease digestion. In control experiments, sonically treated cell lysates were incubated under the same conditions as in the protease digestion but without the protease and fractionated on a gradient. In such experiments, 6S RNA moved ahead of 10S RNA, which suggests that under the conditions employed, the cellular nucleases and proteases did not digest the ribonucleoprotein particle containing the 6S RNA.

DISCUSSION

Our studies indicate the presence of three stable RNA species in *Escherichia coli* in addition to rRNA and tRNA. They are 4.5S, 6S, and 10S RNA. These are small stable RNAs which can be seen in long-term-labeled cells.

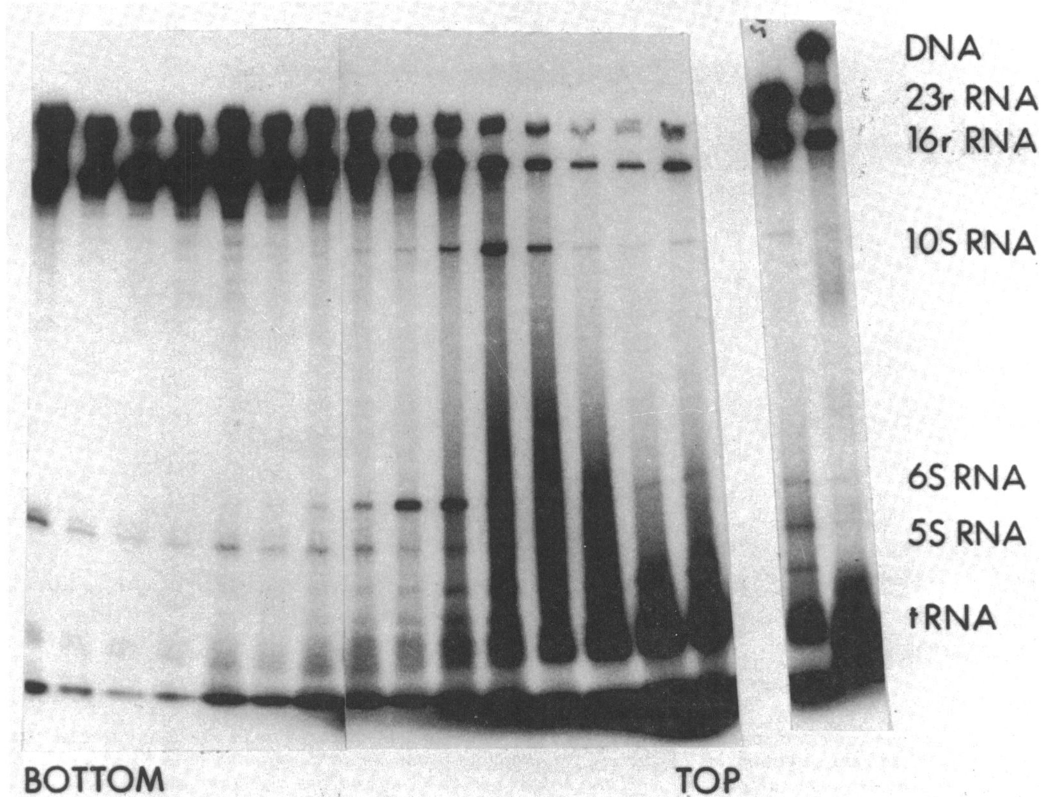

FIG. 5. *Sonically treated, ^{32}P-labeled cell lysate was fractionated in a 10 to 30% sucrose gradient, and the fractions were analyzed on a 5%/12% gel. On the right, the two lanes represent the material before fractionation on the gradient (lane before last) and the pellet obtained after centrifugation of the sonically treated material containing the DNA (last lane; see reference 5). The first and last fractions of the gradient are not shown in the figure. LPS, Lipopolysaccharides; PL, phospholipids.*

They seem to be cytoplasmic RNAs since they are found in the postribosomal supernatant. The presence of 4.5S and 6S RNA as bona fide components of *E. coli* RNA has been shown previously (4, 7-9, 11). Here the 10S RNA is introduced as a third component of such RNA. This component is clearly RNA, for it is labeled with [^{14}C]uracil and is digested by pancreatic RNase. It is always present in long-term-labeled cells of all *E. coli* K-12 strains tested. These three RNAs were also observed in an *E. coli* B strain.

The fact that all the molecules in the 10S region were stable in pulse-chase experiments, whereas variable stability was observed after RNA synthesis was blocked by rifampin, suggests that caution is to be exercised when stability of RNA is measured by decay after RNA synthesis is blocked.

In the region of the gel where the 10S RNA was found, Ilgen et al. (14) identified a number of tRNA precursors in an RNase P^- strain. Since 10S RNA was rather stable, it could not be a precursor to any other molecule. However, the possibility that it contained tRNA sequences which for some reason could not be processed is not excluded by the experiments presented here.

Using the known molecular weights of 6S and 4.5S RNA, we estimated their abundance per genome to be 1,000 to 1,500 molecules or close to one-fourth the number of ribosomes. The physiological functions of these RNAs, present in considerable abundance, are not known. Pettijohn and Hecht (17) have suggested that RNA molecules are involved in maintaining the DNA of *E. coli* in a tightly folded nucleoid structure. Our experiments with purified nucleoid fractions indicate that 6S, 4.5S, and 10S RNA are not present in the nucleoid preparation. Therefore, it is unlikely that any of these three RNAs is involved in maintaining the DNA in its tightly folded conformation.

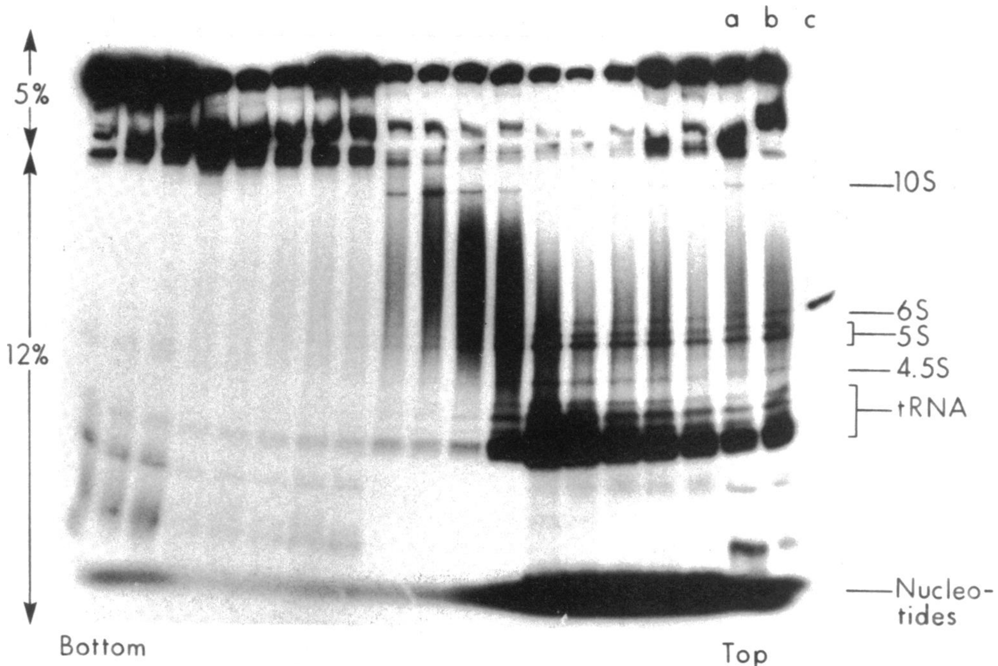

FIG. 6. *Protease-digested sonically treated material. Material was prepared as in Fig. 5 and treated with protease prior to centrifugation in the sucrose gradient. As a marker to distinguish 6S RNA from the 5S RNA bands which became dissociated from the polysomes after the protease digestion, purified 6S RNA is shown in lane c. The curving of the purified 6S RNA band was caused by the edge effect which occurs in slab gels. Lanes a and b contained total sonically treated material with (a) and without (b) protease treatment. In Fig. 5 and 6, where the cell content could not be directly subjected to a hot SDS-containing buffer, some smearing on the gel which resulted from the degradation of RNA was inevitable.*

The behavior of 6S RNA in sucrose gradients indicates that it exists in a small RNP particle in the cell, whereas 4.5S and 10S RNA do not. 10S and 4.5S RNA served as markers in determining the positional changes of 6S RNA in the gradient after various treatments, since their relative positions did not change. All the treatments that were designed to abolish RNA-protein interactions resulted in changing the position of 6S RNA in the gradient, whereas none of these treatments affected the position of 10S or 4.5S RNA. Coupled with the result of the gradient experiments is the fact that 6S RNA is not as stable in vitro as it is in vivo. When dissociated from proteins, purified 6S RNA is much less stable than 4S RNA, and about as stable as 5S RNA. These data suggest that 6S RNA exists in the cell in a ribonucleoprotein particle which we call the "minisome." The association of 6S RNA with proteins seems to render this molecule its unusual stability in vivo. This is the first time that an RNP particle other than ribosomes has been suggested in *E. coli.* In eukaryotic cells, a number of nuclear RNP complexes which contain small stable RNAs are known to exist (18).

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