Initiation of ribosome degradation during starvation in *Escherichia coli*

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ABSTRACT

Ribosomes are known to be degraded under conditions of nutrient limitation. However, the mechanism by which a normally stable ribosome becomes a substrate for the degradation machinery has remained elusive. Here, we present *in vitro* and *in vivo* data demonstrating that free ribosome subunits are the actual targets of the degradative enzymes, whereas 70S particles are protected from such degradation. Conditions that increase the formation of subunits both *in vitro* and *in vivo* lead to enhanced degradation, while conditions favoring the presence of intact 70S ribosomes prevent or reduce breakdown. Thus, the simple formation of free 50S and 30S subunits is sufficient to serve as the initiation mechanism that allows endoribonuclease cleavage and subsequent ribosome breakdown.

Keywords: rRNA; endoribonuclease; ribosome subunits

INTRODUCTION

It is known that ribosomes in *Escherichia coli* are degraded under certain physiological conditions (Deutscher 2003). Typically, such degradation is associated with conditions in which nutrient sources are lacking, such as starvation for carbon (Jacobson and Gillespie 1968), nitrogen (Ben-Hamida and Schlessinger 1966), phosphate (Maruyama and Mizuno 1970), or magnesium ions (McCarthy 1962). Degradation of ribosomes under starvation conditions could enable the bacterium to gain access to the large store of potential nutrients that are present in these macromolecular structures. However, details of the pathway(s) by which ribosomes are degraded, identification of the enzymes involved, and how the process is initiated have remained elusive.

In early studies, it was proposed that rRNA within ribosomes would first be cleaved endonucleolytically, generating large rRNA fragments that subsequently would be degraded to mononucleotides by exoribonucleases (Kaplan and Apirion 1975). Although attempts were made to

identify the enzymes involved, the lack of knowledge at that time of the full repertoire of *E. coli* RNases made this difficult to accomplish (Kaplan and Apirion 1974, 1975; Cohen and Kaplan 1977). The proposed model also failed to address what signals are responsible for initiation of ribosome degradation; namely, how a normally stable ribosome becomes a substrate for the degradation machinery. Several possibilities, alone or in combination, may be envisaged. There may be a new gene product generated during starvation that destabilizes the ribosome, rendering it susceptible to degradation. Second, there could be a conformational change in the ribosome that makes it more labile and sensitive to ribonuclease activity. Third, under starvation conditions, more ribosomes may be present that are not actively translating and, as a consequence, become substrates for degradation.

The studies presented here support the last possibility. Using both *in vitro* and *in vivo* assays, we show that 70S ribosomes are resistant to degradation but that 50S and 30S ribosome subunits are susceptible. Thus, conditions *in vivo* that increase the amount of ribosome subunits lead to elevation of ribosome degradation. We also provide evidence for the involvement of endoribonucleases in initiating the process of ribosome degradation by cleavage of the rRNA present in the ribosome subunits. Endoribonuclease action leads to the accumulation of discrete rRNA fragments *in vivo* that presumably are degraded further by exoribonucleases.

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RESULTS

Degradation of ribosomes in vitro

Ribosomes generally are quite stable in cell extracts provided that the nonspecific endoribonuclease, RNase I, is not present (Deutscher 2003, and references therein). In an attempt to identify factors that might affect ribosome stability, we first examined whether alteration of structure influenced ribosome degradation in vitro. To do this, we varied the Mg^{2+} concentration, an ion known to affect ribosome structure (Gorisch et al. 1976). A representative experiment is shown in Figure 1. Panel A shows the degradation of 23S and 16S RNAs in ^{32}P -labeled ribosomes upon incubation with an extract from cells lacking RNase I carried out at 1, 3, or 5 mM Mg^{2+} ; the data are quantitated in Panel B. At 1 mM Mg^{2+} , both 23S and 16S rRNA were extensively degraded, amounting to $\sim 65\%$ of each RNA species in 1 h. Upon increasing the Mg^{2+} concentration to 3 mM, degradation was impaired, particularly for 23S RNA.

FIGURE 1. Effect of varying $[Mg^{2+}]$ on ribosome degradation in vitro. Assays were performed as described in Materials and Methods with $[Mg^{2+}]$ as indicated. (A) Samples were analyzed by gel electrophoresis. (Lanes 1–3) 1 mM Mg^{2+} ; (lanes 4–6) 3 mM Mg^{2+} ; (lanes 7–9) 5 mM Mg^{2+} . Lanes 1,2,4,5,7,8 contain no cell extract. Lanes 3,6,9 contain 10 μ g cell extract. Lanes 1,4,7 are zero time controls. All other lanes were incubated for 60 min at 37°C. (B) Densitometry was used to compare the band intensities of samples incubated with extract to the intensities of the no extract controls to obtain the percent degradation for each RNA. A representative experiment is presented. Although the absolute level of degradation varied somewhat among multiple experiments, the greatly increased degradation as the $[Mg^{2+}]$ is lowered was observed reproducibly.

Thus, degradation of 16S RNA in the 30S subunit was reduced to $\sim 40\%$, and that of 23S RNA in the 50S subunit was reduced to $<20\%$. At 5 mM Mg^{2+} , rRNA degradation is further decreased, amounting to only $\sim 10\%$ of each RNA. These data show that the Mg^{2+} concentration can have a dramatic effect on ribosome stability in a cell extract.

$[Mg^{2+}]$ is known to have multiple effects on ribosome structure. Thus, as $[Mg^{2+}]$ is lowered, levels of individual subunits increase and the structures of the subunits become less compact (Gesteland 1966, Gorisch et al. 1976). To help distinguish whether formation of ribosome subunits or looser ribosome structure might be the explanation for the decreased stability of rRNA as the $[Mg^{2+}]$ is lowered (Fig. 1), 50S and 30S subunits were isolated and their stability was directly examined in extracts at both 5 mM and 1 mM Mg^{2+} and compared to that of 70S ribosomes. The data in Figure 2A show that even at 5 mM Mg^{2+} the RNA in isolated subunits could be degraded by a cell extract, in contrast to 70S ribosomes, which were relatively stable, as already shown in Figure 1. Quantitation of these data (Fig. 2B) showed that at 5 mM Mg^{2+} , subunits were approximately fivefold more sensitive to degradation compared with 70S ribosomes. At 1 mM Mg^{2+} , also quantitated in Figure 2B, degradation of the subunits was even more extensive, amounting to 60% and 80% for 50S and 30S subunits, respectively. From these data, it is evident that subunits are inherently more sensitive to degradation, and that the increased breakdown seen at 1 mM Mg^{2+} in Figure 1 was at least partly due to their formation at this $[Mg^{2+}]$. Second, the fact that the isolated subunits were even more sensitive to degradation at 1 mM than at 5 mM Mg^{2+} suggests that loosening subunit structure may also play a role. Considering that these experiments were carried out with crude extracts, other factors may contribute to the degradation as well. Nevertheless, these data focused our attention on the increased sensitivity of ribosome subunits.

Ribosome degradation in vivo

Ribosomes are known to be stable during growth but are degraded under certain stress conditions such as nutritional deprivation (Deutscher 2003). To extend our studies to ribosome degradation in vivo, we focused on carbon starvation. In order to measure ribosome degradation during such a stress, cells were grown for many generations in M9/glucose supplemented with $[^3H]$ -uridine such that all cellular RNA would be labeled (rRNA represents $\sim 85\%$ of total RNA under these conditions) (Bremer and Dennis 1996). At mid-exponential phase, cells were collected, washed in M9 salts to remove residual glucose and $[^3H]$ uridine, and resuspended in M9 medium in the presence or absence of 0.4% glucose. At various times, a portion of each culture was removed and acid-soluble radioactivity determined.

A typical experiment is presented in Figure 3. The data show that cells in the presence of glucose degraded

FIGURE 2. Comparison of degradation of ribosomes and ribosome subunits in vitro. (A) Assays were performed and analyzed as described in Materials and Methods at 5 mM Mg^{2+} . (Lanes 1–3) 70S ribosomes; (lanes 4–6) 50S subunits; (lanes 7–9) 30S subunits. Lanes 1,2,4,5,7,8 contain no cell extract. Lanes 3,6,9 contain 10 μ g wild-type (WT) extract. Lanes 1,4,7 are zero time controls. All other lanes were incubated for 45 min at 37°C. (B) Densitometry was used to compare band intensities of samples incubated with extract to the intensities of the zero time controls to obtain a percentage of degradation for each rRNA in 70S ribosomes or in ribosome subunits. Quantitation is also shown for a similar experiment with ribosome subunits at 1 mM Mg^{2+} . The number *above* the bars is the Mg^{2+} concentration used.

pre-existing RNA very slowly, or not at all, during a 3-h period. In contrast, imposition of carbon starvation led to appreciable RNA degradation amounting to ~12% in 3 h. Degradation continued for at least 24 h reaching close to 60% acid soluble in this time period (data not shown). Based on the amount of acid soluble radioactivity released, it is evident that destruction of ribosomes is occurring (additional evidence is presented below). Thus, this simple protocol provides a useful assay to follow the degradative process in vivo. It is important to note that this procedure measures only the smallest degradation products, i.e., mononucleotides and oligonucleotides, and this was confirmed by paper chromatography of the acid soluble material (data not shown). Large fragments generated from rRNA breakdown were detected by other methods (see below). It should also be noted that there was essentially no change in the number of viable cells in the absence of glucose based on platings for periods of up to 6 h.

Analysis of ribosome degradation during carbon starvation

To more directly examine the fate of rRNA during carbon starvation, RNA was extracted from cells placed in the

presence or absence of glucose and analyzed using gel electrophoresis. As shown in Figure 4, cells grown in the presence of glucose contained primarily 23S and 16S rRNA. However, in the absence of glucose, these RNAs were almost completely absent, and discrete fragments of varying sizes accumulated. These data show that rRNAs are extensively degraded during starvation and that the action of an endoribonuclease(s) likely initiates breakdown of the rRNAs. This was confirmed in a separate experiment by Northern analysis (Fig. 5) using probes against the 3' ends of 23S and 16S rRNAs. As can be seen, large fragments were generated from the 3' end of each RNA that could only arise by endonucleolytic cleavage. Similarly, large fragments of each rRNA were observed with 5' probes (data not shown).

Ribosomes were also examined using sucrose gradient analysis. As shown in Figure 6, in the presence of glucose, cells contained predominately 70S ribosomes with only a small amount of 50S and 30S ribosome subunits present. However, after removal of glucose, there was a dramatic loss of ribosomes and a large increase in degraded material at the top of the gradient. Moreover, while 70S ribosomes decreased substantially (~50%), 50S and 30S subunits were eliminated entirely. These data confirm that ribosomes break down during carbon starvation, and they are consistent with the conclusion from the in vitro experiments that 50S and 30S subunits are the immediate substrates for the degradative process.

Effect of rifampicin on ribosome degradation

To confirm the conclusion that ribosome subunits are the substrates for the degradation machinery and also to examine whether synthesis of a new gene product might be required to signal the initiation of ribosome degradation,

FIGURE 3. Assay of ribosome degradation during starvation for glucose in vivo. Wild-type cells were grown in the presence of [3H]-uridine, followed by cell collection and resuspension in M9 medium $\pm$ glucose, as described in Materials and Methods. Samples were taken at 0, 1, 2, and 3 h. Degradation (%) was determined by comparing acid-soluble radioactivity to total radioactivity in the sample in the presence (■) or absence (□) of glucose.

FIGURE 4. Analysis of RNA from cells undergoing carbon starvation. Total RNA was extracted from cells placed in the presence or absence of glucose for 6 h, and RNA was quantified by A_{260} measurement as described in Materials and Methods. Equal amounts of RNA were loaded onto a 3% denaturing polyacrylamide gel and subjected to electrophoresis. RNA was visualized by staining with ethidium bromide and exposure to a UV lamp.

cells were starved *in vivo*, as described above, but with the addition of rifampicin, an antibiotic known to inhibit initiation of transcription. If a new gene product were necessary to initiate the degradative process, it might be expected that degradation would decrease or be eliminated upon rifampicin treatment. However, as shown in Figure 7A, degradation actually increased $\sim 40\%$ in comparison to cells starved for glucose in the absence of rifampicin. These data indicate that synthesis of the mRNA for a new gene product is not responsible for the initiation of ribosome degradation. Moreover, they are consistent with degradation occurring at the subunit level, as translation should be reduced in the presence of rifampicin due to decreased availability of mRNA, and more ribosome subunits should be present than in the absence of rifampicin.

Effect of neomycin on ribosome degradation

To directly examine whether the level of ribosome subunits affects degradation, starvation was carried out in the presence of neomycin, an antibiotic recently shown to inhibit subunit dissociation *in vivo* (Borovinskaya et al. 2007). The data in Figure 7B show that in the presence of neomycin, ribosome degradation was inhibited by more than 60%. Thus, maintaining ribosomes as 70S particles and thereby decreasing the amount of ribosome subunits dramatically decreases the degradation of ribosomes during carbon starvation. These data support the conclusion that

ribosome subunits are the immediate substrates for the initiation of ribosome breakdown.

DISCUSSION

Although the degradation of ribosomes during periods of cellular stress has been known for many years, the signal that initiates this process has until now remained elusive. Based on *in vitro* and *in vivo* analysis of this process, we propose a simple mechanism to explain how the normally stable ribosomes in growing cells become substrates for degradation. During starvation, ribosome subunits that typically are rapidly recycled into translating ribosomes during exponential growth remain idle because as the growth rate decreases, the translation machinery is less actively engaged. As shown here, ribosome subunits are inherently much more sensitive to degradation. Hence, degradation is built into the system itself, and synthesis of new components is not required. Ribosomes not engaged in translation, and consequently present as subunits, are by their nature more sensitive to degradation, and any physiological condition increasing the amount of subunits will, therefore, lead to enhanced degradation.

Support for this model comes from both *in vitro* and *in vivo* experiments. *In vitro*, degradation of ribosomes was greatly accelerated at lower $[Mg^{2+}]$, a condition that favors separation into subunits. Moreover, direct assay of ribosome subunits showed that they were much more sensitive to breakdown by a cell extract than were 70S ribosomes. Use of an *in vivo* assay for ribosome degradation confirmed these observations. Treatment of starving cells with rifampicin revealed that transcription was not necessary for ribosome degradation, and in fact, it increased the level of degradation. These data indicate first that a new gene

FIGURE 5. Northern blot analysis of rRNA isolated from 70S ribosomes. RNA (1 μ g) extracted from cells grown in the presence (+) or absence (–) of glucose was resolved on 1.5% agarose gels, stained with ethidium bromide (panel A), transferred to nylon membranes, and probed with 23S 3'-end specific or 16S 3'-end specific (panel B) probes, as described in Materials and Methods. Arrows indicate the positions of major new bands. Band size was estimated from the positions of RNA standards visualized by ethidium bromide staining. Note that a product from 23S degradation migrates in the position of 16S RNA.

FIGURE 6. Analysis of ribosomes from cells undergoing carbon starvation. Extracts of cells grown in the presence or absence of glucose for 6 h were run on sucrose gradients for 15 h at 15,000 rpm. Equal amounts of RNA were added to the gradients. Fractions of 0.5 mL were collected and were analyzed for RNA content by A_{260} . Presence (■) or absence (□) of glucose. Note the break in the scale for the top fraction of the no glucose profile.

product is not necessary for the degradation process to initiate. Second, the increased level of ribosome degradation is consistent with the mode of action of rifampicin. In its presence, fewer transcripts would be available for recycling of ribosome subunits, leading to more substrate that can be acted on by the degradative machinery. In contrast, treatment with neomycin, as a consequence of its anti-dissociation properties, would be expected to maintain the 70S particle, thereby decreasing the amount of available substrate and leading to decreased degradation, as was found.

Most importantly, this model fits very well with our increased understanding of ribosome structure (Schuwirth et al. 2005; Korostelev and Noller 2007; Steitz 2008). The high-resolution X-ray structures show that the majority of exposed rRNA in ribosomes is found on the subunit interfaces. Thus, the action of ribonucleases on rRNA would be favored when the 50S and 30S subunits are dissociated, whereas association in a 70S particle during translation would serve to protect the exposed rRNA from nuclease attack.

Taken together, all of this information lends strong support to the conclusion that free subunits are the immediate substrates for ribosome degradation. They provide a simple, yet elegant mechanism, with which to signal the initiation of the degradative process. Any ribosome subunit not actively recycled into 70S ribosomes potentially becomes a target for endoribonuclease action and ultimately degradation.

The model also raises a number of interesting questions for future studies. For example, what prevents the degradative machinery from acting on ribosome subunits as they recycle during the normal translation process in growing cells? One hint regarding this question comes from the work of Davis et al. (1986). They showed that translation initiation

factors were not detectable during starvation. Perhaps, these and/or other protective factors could bind the subunits and inhibit nuclease action even before the reinitiation process is complete and 70S ribosomes have re-formed.

Also of considerable interest is the identification of the RNases responsible for ribosome degradation. Based on the data presented, we believe that an endoribonuclease(s) is responsible for initiating the degradative process by cleaving RNA at the subunit interfaces. Additional endoribonuclease and exoribonuclease action on the initial rRNA fragments then generates the oligoribonucleotide and mononucleotide products that we observe in the acid-soluble fraction. Studies are now in progress using purified enzymes and RNase-deficient mutant cells to identify the RNases responsible for the breakdown of rRNA.

MATERIALS AND METHODS

Bacterial strains

Strains CA244 I⁻ and MG1655 I⁻ were considered to be wild type for this study. The RNase I⁻ derivatives were constructed by recombineering (Datsenko and Wanner 2000; Datta et al. 2006), and were confirmed by PCR and direct assay for RNase I.

Preparation of cell extracts

Cells grown to late exponential phase ($A_{600} \sim 1.0$) in yeast/tryptone medium at 37°C were cooled on ice for 20 min and were collected by centrifugation in a Sorvall SS34 rotor for 10 min at 5000 rpm at 4°C. The resulting cell pellets were stored at -80°C. Prior to use, cells were resuspended in 25 mM Tris-Cl (pH 7.6) 400 mM KAc, 1 mM dithiothreitol (DTT) and ruptured by two passes through an Aminco French press at 18,000 p.s.i. Cell debris was removed by centrifugation in a Sorvall SS34 rotor for 15 min at 15,000 rpm at 4°C. The protein concentration of the clarified lysate was measured by the Coomassie method (Bradford 1976). Portions were frozen in liquid nitrogen and stored at -80°C.

Preparation of [³²P]-labeled ribosomes

Strain MG1655 I⁻ was grown to mid-exponential phase in LP medium (100 mM Tris-Cl at pH 7.6, 85 mM NaCl, 20 mM KCl, 18.6 mM NH₄Cl, 0.2% casamino acids, 0.2% bactopectone, 1 mM MgSO₄, 0.2% glucose) supplemented with 1 μ Ci/mL of [³²P]-inorganic phosphate (Perkin-Elmer). Cultures were cooled on ice for 20 min, and cells were collected by centrifugation in a Sorvall GSA rotor for 10 min at 5000 rpm at 4°C. Ribosome isolation was adapted from Powers and Noller (1991). Cell pellets were washed once in ice-cold buffer A (50 mM Tris-Cl at pH 7.6, 10 mM MgCl₂, 0.1 M NH₄Cl, 6 mM 2-mercaptoethanol, 0.5 mM EDTA) and stored at -80°C. Cells were thawed on ice, resuspended in buffer A, and lysed by two passes through an Aminco French press at 18,000 p.s.i. Two units of DNase I (New England Biolabs) were added to the lysate. The lysate was then clarified by two consecutive centrifugations in a Sorvall SS34 rotor at 15,000 rpm for 15 min at 4°C. The supernatant fraction was brought to 1 M NH₄Cl, layered on buffer A containing 1 M NH₄Cl and 18%

FIGURE 7. Assay of ribosome degradation during starvation for glucose in the presence of rifampicin or neomycin in vivo. (A) Experiments were carried out as in Figure 3 except that rifampicin (100 $\mu\text{g}/\text{mL}$) was added to one culture starved for glucose. (B) Experiments were carried out as in Figure 3 except that neomycin (500 $\mu\text{g}/\text{mL}$) was added to one culture starved for glucose.

sucrose, and spun in a Beckman ultracentrifuge at 44,000 rpm for 19 h in a Beckman Ti 70.1 rotor at 4°C. The ribosome pellets were resuspended in buffer B (50 mM Tris-Cl at pH 7.6, 10 mM MgCl_2 , 0.1 M NH_4Cl , 6 mM 2-mercaptoethanol) by gentle rocking overnight at 4°C and were stored in small portions at -80°C . Prior to use, ribosomes were layered on a 14%–32% sucrose gradient in buffer B plus 1 M NH_4Cl and centrifuged in a Beckman SW28 rotor for 19 h at 21,000 rpm to remove residual RNases. Fractions were collected and quantified by A_{260} measurement and liquid scintillation counting. Purified ribosomes were stored in small portions at -80°C .

Preparation and electrophoresis of RNA

RNA was extracted by phenol/chloroform treatment of 70S particles purified on sucrose gradients (see below) and precipitated with ethanol. Samples were loaded either onto a 3% denaturing polyacrylamide gel or a 1.5% agarose gel; analyzed by electrophoresis at 2 V/cm for ~ 2 h or at 5 V/cm for 1 h, respectively; and visualized by ethidium bromide staining.

Northern blot analysis

rRNA (1 μg) was resolved on a 1.5% agarose gel in 1xTAE buffer (40 mM Tris-acetate, 1 mM EDTA) and transferred to a nylon membrane by downward capillary transfer for 3 h using 1xTAE as the transfer solution. DNA oligonucleotide probes complementary to either the 3'-end of the 16S RNA (5'-aaggaggatccaaccgca-3') or the 3'-end of the 23S RNA (5'-aaggttaagcctcaggttc-3') were [^{32}P]-labeled at their 5'-ends by T7 polynucleotide kinase. Probes were allowed to anneal to the transferred RNA by overnight incubation in ExpressHyb hybridization solution (Clontech), and the detected bands were visualized by PhosphorImager (Molecular Dynamics) analysis.

In vitro assays for ribosome degradation

Reaction mixtures (100 μL) contained 25 mM Tris-Cl (pH 7.6), 400 mM KAc, 1 mM DTT, 2 μg [^{32}P]-labeled ribosomes, and 10 μg cellular extract. Samples usually were incubated for 60 min at 37°C. For subsequent electrophoresis, 10 μL were removed and mixed

with 10 μL of loading buffer (10 mM Tris-Cl at pH 7.6, 10 mM EDTA, 1% SDS, 40% glycerol, 0.1% diethylpyrocarbonate [DEPC], 0.25% bromophenol blue), vortexed, and boiled for 2 min before loading on a 3% denaturing polyacrylamide gel. Samples were subjected to electrophoresis at 200 mA for 3 h. The gel was then placed on Whatman filter paper and directly used for autoradiography.

In vivo assay for ribosome degradation

A single colony was inoculated into 2 mL of M9/0.2% glucose medium. After overnight growth, 100 μL was inoculated into 100 mL of M9/0.2% glucose supplemented with 1 $\mu\text{Ci}/\text{mL}$ of [^3H]-uridine (GE) and 0.1 mM uridine. Cultures were grown to mid-exponential phase. Cells were collected by centrifugation for 10 min in a Sorvall SS34 rotor. The cell pellet was washed once in M9 salts and resuspended in 5 mL of M9 salts. Half was inoculated into a culture of 45 mL of M9/0.4% glucose and 0.1 mM uridine, while the rest was inoculated into 45 mL of M9 salts and 0.1 mM uridine, lacking glucose. In some assays, antibiotics were also added at the indicated concentrations. A_{600} readings were taken to monitor growth. At indicated times, 500 μL portions were removed from the culture and treated with 4 M formic acid (Cohen and Kaplan 1977). After 15 min on ice, samples were centrifuged at maximum speed for 15 min in a Fisher bench top microcentrifuge at 4°C. Half of the supernatant fraction was removed and neutralized with 1 M Tris. Ten milliliters of scintillation fluid was added, and samples were counted in a scintillation counter to determine acid-soluble radioactivity.

Sucrose gradient analysis

Cells were grown as above for the in vivo assay except that [^3H]-uridine was omitted. After 6 h of incubation, cell extracts were made as described above. RNA amounts were determined by A_{260} measurement. Equal amounts of RNA were layered onto 5%–20% sucrose gradients containing 20 mM Tris-Cl (pH 7.6), 15 mM $\text{Mg}(\text{OAc})_2$, 100 mM NH_4Ac , 1 mM DTT in DEPC-treated H_2O (Wada et al. 2000) and centrifuged for 15 h at 15,000 rpm in a Beckman SW41 rotor at 4°C. Gradients were analyzed as described in the section for obtaining [^{32}P]-labeled ribosomes.

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