Multiple Exoribonucleases Catalyze Maturation of the 3’ Terminus of 16S Ribosomal RNA (rRNA)*

Processing of ribosomal RNA (rRNA) precursors is an important component of RNA metabolism in all cells. However, in no system have we yet identified all the RNases involved in this process. Here, we show that four 3’→5’-exoribonucleases, RNases II, R, and PH, and polynucleotide phosphorylase (PNPase), participate in maturation of the 3’ end of 16S rRNA. In their absence, 16S precursor molecules with 33 extra 3’-residues accumulate; however, the presence of any one of the four RNases is sufficient to allow processing to occur, although with different efficiencies. Additionally, we find that in the absence of 3’ maturation, 5’ processing proceeds much less efficiently. Moreover, mutant 30S particles, containing immature 16S rRNA, form 70S ribosomes very poorly. These findings, together with the earlier discovery that RNases E and G are the 5’-processing enzymes, completes the catalogue of RNases involved in maturation of *Escherichia coli* 16S rRNA.

Ribosomal RNAs (rRNAs) are initially synthesized as precursor molecules that subsequently are processed to the mature forms that function in protein synthesis. However, despite extensive study over many years, we still cannot fully describe the rRNA maturation pathways in any biological system, nor how the maturation events are coordinated with the process of ribosome assembly (1). In *Escherichia coli*, maturation is initiated by RNase III cleavage of the rRNA transcript to generate precursors of the three rRNAs, 16S, 23S, and 5S rRNA (2, 3). The extra 5’- and 3’-residues from each of these precursors are then removed by the action of multiple RNases (1, 4).

In the case of 16S rRNA, the extra 115 nt at the 5’ end of the molecule are removed in a two-step process that involves cleavage by the endoribonuclease RNase E, at a position 66 nt upstream of the 5’ end, followed by the action of a second endoribonuclease RNase G, which cleaves at the mature 5’ terminus (5, 6). Thus, 5’ processing of 16S rRNA involves an ordered sequence of events, and the RNases involved have been identified.

In contrast, very little is understood about maturation of the 3’ terminus of 16S rRNA. It is known that 3’ maturation can proceed in the absence of 5’ maturation (5). It is also known that the 33 extra 3’-residues are removed rapidly such that no intermediates with less than 33 nt are observed. This finding suggested that 3’ maturation might be an endonucleolytic process, although the action of a 3’-5’ progressive exoribonuclease is also possible. In fact, in *Pseudomonas syringae*, the processive exoribonuclease RNase R is required for 3’ maturation of 16S rRNA in the cold, although RNase R is not essential at room temperature (7). An exoribonuclease has also been implicated in the 3’ maturation of rRNAs in chloroplasts of *Arabidopsis thaliana* (8). However, it is not clear whether these are unusual examples or whether exoribonucleolytic processing of the 3’ terminus of 16S rRNA is a widespread phenomenon.

In this study, we examine 3’ processing of 16S rRNA in *E. coli*. We show that any one of several, known exoribonucleases can carry out removal of the 33 extra 3’-nucleotides. Most important are the three 3’-5’ progressive exoribonucleases, RNase II, RNase R, and polynucleotide phosphorylase (PNPase). However, in their absence, 3’ processing continues at a slow rate, largely due to RNase PH. Mutant strains lacking all four exoribonucleases are essentially unable to generate mature 16S rRNA. Such cells accumulate precursors that retain not only the 33-nt 3’ trailer sequence, but also their 5’ leader. These data demonstrate that, as with maturation of tRNAs in *E. coli* (9), 3’ maturation of 16S rRNA makes use of multiple exoribonucleases.

**MATERIALS AND METHODS**

*Bacterial Strains—* *E. coli* MG1655*(seq) I− was used as wild type for this study (10). MG1655 I− was used as the RNase PH− strain into which RNase R, RNase II, and PNPase mutations were introduced as described previously (10).

*Site-directed RNase H Cleavage—* Total RNA was isolated using a hot phenol method (11) and subjected to RNase H assay followed by Northern blot analysis (5, 12). The chimera C16S3 (5’-CCdCdGdAAGGUUAAGCCUCGCG-C3’) was used for 3’ end cleavage of 16S rRNA, and cleavage products were visualized by using a probe complementary to residues 1502–1523. Chimera C1655 (5’-CAdTdTdGUUAGGCCUCGCG-C3’) was used for 5’ end cleavage and visualized by a probe complementary to residues 18–36.

*Sucrose Gradient Analysis of Ribosomes—* Cells were grown at 37 °C until the A600 = 0.4 and then shifted to 44 °C for 1 h.

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1 To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of Miami Miller School of Medicine, P. O. Box 016129, Miami, FL 33101. Tel.: 305-243-3150; Fax: 305-243-3955; E-mail: mdeutsch@med.miami.edu.

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Cells were collected by centrifugation and resuspended in buffer A (10 mM Tris-Cl, pH 7.5, 10 mM MgCl₂, 60 mM KCl, and 1 mM DTT) containing 2 mg/ml lysozyme, and the lysate was clarified by sonication followed by centrifugation. The supernatant fraction was spun at 55,000 rpm for 2 h. Ribosome pellets were resuspended and layered on a 10–30% sucrose gradient in buffer B (10 mM Tris-Cl, pH 7.5, 60 mM NH₄Cl, and 1 mM DTT) containing 0.1 mM MgCl₂ for analysis of 30S and 50S subunits. For analysis of 70S particles, ribosomes were layered on a 14–40% sucrose gradient made in buffer B containing 10 mM MgCl₂. The gradient samples were centrifuged for 19 h at 21,000 rpm. Fractions were collected and quantified by A₂₆₀ measurement. The ribosomal fractions were subjected to Northern blot analysis using probe P16S3 (5'-TGTGAGCACTGCAAAGAACGC-3') for detecting 3’ precursor 16S rRNA and probe 16S (5'-CCATGGTGTGACGGGCGGTG-3') for detecting mature 16S rRNA.

RESULTS

Exoribonucleases Participate in 3’ Processing of 16S rRNA—To assess whether exoribonucleases might play a role in 16S rRNA processing, we made use of mutant strains lacking one or more known exoribonucleases. Total RNA was isolated from each of 10 strains and examined by Northern blot analysis using one probe directed against the mature portion of 16S rRNA and a second probe directed against the 33-nt precursor region. From the ratio of precursor to mature signal, we could determine whether any mutant strain accumulated more precursor than wild type cells. Based on this preliminary screening (data not shown), we observed that cells deficient in certain combinations of exoribonucleases showed an elevated level of precursor. These data focused our attention on the processive exoribonucleases, RNase II, RNase R, and PNPase, as well as on RNase PH. No evidence was obtained for the involvement of RNases D, BN, or T. Nevertheless, these data suggested that 3’ processing of 16S rRNA might require certain exoribonucleases and that more detailed examination was warranted.

Four Known Exoribonucleases Contribute to 3’ Processing of 16S rRNA—To confirm the involvement of the exoribonucleases identified by the screening assay, we constructed a quadruple mutant strain lacking RNases II, R, and PH and containing a temperature-sensitive PNPase. This was necessary because double mutant strains lacking RNase II and PNPase (13) or RNase R and PNPase (14, 15) are inviable, and cells lacking RNase PH and PNPase are slowed in growth (16). The mutant strain was grown at 37 °C and transferred to 44 °C for 1 h to inactivate PNPase such that the maturation of 16S RNA could be examined under conditions in which the activities of four exoribonucleases were lacking. Using RNase H digestion to more easily distinguish precursor from mature 16S rRNA, we compared the amount of precursor in the quadruple RNase mutant strain with that in wild type using Northern blot analysis. The data in Fig. 1A show that there is essentially no precursor (2%) in wild type cells (lane 1), whereas in cells lacking RNases II, R, and PH and PNPase, close to 90% of 16S rRNA contains a 33-nt 3’ extension (lane 2). These data demonstrate that exoribonucleases catalyze 3’ maturation of 16S rRNA, and because no intermediate between the +33 and mature species is evident, demonstrate that they act processively on the 33-nt extension.

To assess the role of each enzyme in the process, we utilized four triple mutant strains in which the activity of one of the four missing RNases was restored. These strains were compared with wild type and with the quadruple RNase-deficient mutant strain (Fig. 1A). The data show that reintroduction of any one of the four exoribonucleases increased the amount of 3’ mature 16S rRNA as compared with the quadruple mutant strain. RNase II and RNase R were most effective, each restoring 3’ maturation almost completely. PNPase was only slightly less effective. RNase PH, on the other hand, led to only ~50% of the normal amount of 16S rRNA 3’ processing. These findings indicate that four different exoribonucleases each can contribute to 3’ maturation of 16S rRNA and that only in the absence of all four RNases is 3’ maturation largely eliminated.

Essentially identical results were obtained by Northern blot analysis using probes directed against the 3’ precursor region and mature 16S rRNA (Fig. 1B). However, the Northern analysis also revealed that in the most defective mutant strains, those lacking all four exoribonucleases (lane 2) or containing only RNase PH (lane 4), extensive degradation of 16S rRNA occurs. Thus, if maturation is blocked, a previously described quality control process on rRNA becomes prevalent (15), although in the absence of RNase R and PNPase, degradation is not complete, and specific fragments accumulate.

Pulse-Chase Analysis of 3’ Maturation of 16S rRNA—To expand on the steady-state analysis presented in Fig. 1, we carried out pulse-chase experiments to compare the rate of 3’ processing in the quadruple RNase-deficient strain with that in wild type cells. After incubation at 44 °C for 1 h to inactivate PNPase, cells were labeled for 5 min with ³²P followed by the addition of rifampicin to prevent synthesis of additional rRNA...
molecules. Samples were taken over a period of 20 min to measure the rate of conversion of precursor to mature 16S rRNA (Fig. 2). Precursor RNA in wild type cells is so rapidly converted to the mature species that even at the zero time point, 40% of the labeled RNA is already mature, representing RNA molecules that had been transcribed early during the pulse period. In contrast, conversion of precursor to mature 16S rRNA is dramatically slowed in the RNase-deficient strain. Even after 20 min of chase, >70% of the RNA is still present in precursor form. These data confirm that removal of the four exoribonucleases, RNase II, RNase R, and PNPase, and RNase PH, dramatically decreases 3’ processing of 16S rRNA. It is not yet clear whether the very slow rate of processing that remains is due to incomplete inactivation of the temperature-sensitive PNPase or to yet another RNase with low processing activity.

**Lack of 3’ Processing of 16S rRNA Affects 5’ Processing**—To determine whether 5’ processing of 16S rRNA is affected by the absence of exoribonucleases that mature the 3’ end, we simultaneously examined both termini of the RNA molecule in wild type and RNase-deficient strains. For this analysis, we used the same RNase H cleavage method as in Fig. 1A, but included as well a 5’-specific chimeric oligonucleotide complementary to residues 40–57. This allowed clear separation of RNA populations containing 115 or 66 extra 5’-residues or mature 5’ ends (Fig. 3). The experiment shown in panel A was carried out as in Fig. 1A, with very similar results for 3’ processing in the various mutant strains. Analysis of the 5’ ends of the 16S rRNA present in each of the strains (Fig. 3B) revealed that the percentage of molecules with unprocessed 5’ termini was essentially the same as those with unprocessed 3’ ends. Moreover, most of the unprocessed 5’ termini contained 115 rather than 66 extra 5’-residues. These data indicate that 5’ maturation is dependent on prior 3’ processing and that in its absence, the initial RNase E cleavage at position +66 (5) is severely inhibited.

**Mutant 30S Subunits Are Not Assembled into 70S Ribosomes**—To determine the fate of unprocessed 16S rRNA precursor, we compared ribosomes isolated from the quadruple RNase-deficient cells with those isolated from wild type (Fig. 4). Both ribosome subunits and intact ribosomes were examined using sucrose gradient sedimentation. At low Mg2+, in which ribosomes are present as subunits, the profiles from the wild type and mutant strains are similar, although the 30S peak in the mutant is slightly broader (Fig. 4A). However, Northern blot analysis revealed that although 30S particles from the wild type are devoid of 16S precursor RNA, those derived from the mutant strain contain a majority of precursor RNA molecules. Note also that the ratio of precursor to mature 16S rRNA differs in the two mutant fractions analyzed, indicating that the mutant 30S peak is heterogeneous. Thus, 30S particles can be assembled even when 16S RNA is not matured, although as shown below, these 30S particles are abnormal.

In contrast to low Mg2+, at 10 mM Mg2+, wild type ribosomes are found primarily as 70S particles (85%) (Fig. 4B). In contrast, <60% of mutant ribosomes are present as 70S particles, and there is a deficit of 30S subunits relative to 50S subunits. This finding is consistent with the considerable degradation of 16S rRNA seen in the RNase-deficient strain. Moreover, Northern blot analysis indicates that the precursor 16S rRNA present in the mutant strain is found largely in the 30S peak. These data support the conclusion that the 30S subunits assembled in the absence of the four exoribonucleases are abnormal, and presumably because they are degraded so rapidly, participate poorly in the formation of 70S particles. The small amount of 16S precursor in the 70S peak likely is due to particles that associated with 50S subunits and thereby escaped degradation.

**DISCUSSION**

The findings presented here indicate that maturation of the 3’ end of 16S rRNA in *E. coli* is carried out by four known 3’→5’-exoribonucleases, and the presence of any one of them is sufficient to enable cell growth. The three processive exoribonucleases, RNase II, RNase R, and PNPase, are most effective, but cells grow even when only RNase PH remains, although considerably more slowly. The fact that each of the four RNases can completely mature the 3’ end of the RNA explains why it has been so difficult to identify the enzyme responsible for 3’
FIGURE 3. Analysis of 3′ and 5′ termini of 16S rRNA in WT and mutant strains. A, 3′ end; B, 5′ end. The same RNA sample was subjected to RNase H analysis using probes described under “Materials and Methods.” M is the position of mature 16S rRNA 3′ or 5′ ends; the precursor fragment with an extra 33 nt is marked as +33. Precursors with 115 or 66 extra nt at the 5′ end are marked as +115 or +66. PNP⁰ is a temperature sensitive mutant of PNPase.

FIGURE 4. Mutant 30S subunits do not assemble into 70S particles. WT and R−PH−II−PNP⁰ ribosomes were prepared as described under “Materials and Methods.” A, sucrose gradient analysis in 0.1 mM MgCl₂ and Northern blot analysis of two fractions from the 30S peaks from WT and mutant cells were carried out as described under “Materials and Methods” using probes complementary to the precursor and mature regions of 16S rRNA. B, sucrose gradient analysis in 10 mM MgCl₂ and Northern blot analysis of fractions across the sucrose gradient. PNP⁰ is a temperature sensitive mutant of PNPase.
processing. Mutants lacking exoribonucleases had previously been examined for defects in 3′ maturation of 16S RNA (1), but in no case were all removed simultaneously. Because elimination of the four RNase activities decreases 16S 3′ maturation by close to 90%, it is clear that they are the main participants in the process.

Based on the information presented here, we now have a fairly complete picture of the maturation pathway for 16S rRNA in *E. coli* (1). Initially, the double strand-specific endoribonuclease, RNase III, cleaves the rRNA transcript in a double-stranded stem generated by complementarity between precursor regions flanking both sides of 16S RNA to generate a 17S precursor molecule containing 115 extra 5′-nucleotides and 33 extra 3′-nucleotides. Then, the 33 extra 3′-nucleotides are removed by any one of four exoribonucleases, RNase II, R, PH, or PNase. Removal of the extra 3′-residues results in the extra 5′-residues becoming single-stranded, which enables the single-strand-specific endoribonuclease, RNase E, to cleave at residue +66 to generate the 16.3S rRNA precursor (5, 6). Cleavage at +66 facilitates the subsequent cleavage by RNase G at the mature 5′ terminus. This sequence of RNA processing events occurs within the context of a preribosomal particle, and the next major challenge is to elucidate how they are coordinated with ribosome assembly.

One interesting question that remains to be answered is how the exoribonucleases identified here digest through the extensive secondary structure that results from the complementarity of the extra 5′- and 3′-residues in the 16S rRNA precursor. Although RNase R and PNase as part of the degradosome have such capability, RNase II does not. Consequently, at least with this exoribonuclease, it is extremely likely that an RNA helicase will be directly involved in 3′ processing of 16S rRNA. Further analysis will determine whether this prediction is borne out.

With the identification of the RNases responsible for 3′ maturation of 16S RNA, we now know how the 3′ termini of essentially all stable RNAs are generated in *E. coli*. This includes 16S (this work), 23S (12, 17), and 5S (18) rRNAs, tRNAs (9), and other small stable RNAs (19). In all cases, removal of the last precursor residues involves the action of an exoribonuclease. This contrasts with other organisms, including other eubacteria, in which endoribonuclease action may occur (e.g. Refs. 20–22). The exoribonucleolytic mode of 3′ processing in *E. coli* raises not only interesting evolutionary questions, but also questions of how the exoribonucleases accurately stop at the mature 3′ position, particularly when some of the RNases are processive enzymes. Undoubtedly, the context of the preribosomal particle is involved, as was previously shown for RNase T action on 5S RNA (18) and 23S RNA (12) precursors, but further study will be required to clarify this for 16S RNA.

Although identification of the RNases involved in 16S RNA processing provides a major advance, it is clear that multiple other factors also participate in ribosome biogenesis. These include ribosomal proteins, rRNA-modifying enzymes, RNA helicases, GTPases, and other proteins of unknown function. Exactly what role, if any, each of these proteins plays in removal of the 33 extra residues at the 3′ end of the 16S rRNA precursor is not yet known, but it is clear that they are necessary to facilitate the ordered sequence of events that make up RNA processing and ribosome assembly.

In a very recent study (23), one protein of previously unknown function, YbeY, known to participate in 3′ processing of 16S rRNA (24), was shown to be an endoribonuclease. As a consequence, it was proposed that YbeY is responsible for removal of most of the 33 extra 3′-residues, although no direct evidence for this assertion was presented (23). Based on our data, we believe it is unlikely that YbeY directly removes extra 3′-nucleotides inasmuch as all 33 residues remain when the exoribonucleases identified here are removed. Thus, although it is clear that YbeY is needed for 3′ maturation of 16S rRNA (24), its actual role remains to be determined.

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