The tRNA processing enzyme RNase T is essential for maturation of 5S RNA

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ABSTRACT  The maturation of 5S RNA in *Escherichia coli* is poorly understood. Although it is known that large precursors of 5S RNA accumulate in mutant cells lacking the endoribonuclease RNase E, almost nothing is known about how the mature 5' and 3' termini of these molecules are generated. We have examined 5S RNA maturation in wild-type and mutant RNase T-deficient cells by Northern blot and primer-extension analysis. Our results indicate that no mature 5S RNA is made in RNase T-deficient strains. Rather, 5S RNA precursors containing predominantly 2 extra nucleotides at the 3' end accumulate. Apparently, these 5S RNAs are functional inasmuch as mutant cells are viable, growing only slightly slower than wild type. Purified RNase T can remove the extra 3' residues, showing that it is directly involved in the trimming reaction. In contrast, mutations affecting other 3' exoribonucleases have no effect on 5S RNA maturation. Approximately 90% of the 5S RNAs in both wild-type and RNase T cells contain mature 5' termini, indicating that 5' processing is independent of RNase T action. These data identify the enzyme responsible for generating the mature 3' terminus of 5S RNA molecules and also demonstrate that a completely processed 5S RNA molecule is not essential for cell survival.

Most, if not all, RNA molecules are initially synthesized as longer precursors that must undergo a series of processing reactions to form the mature, functional form of the RNA (1, 2). Despite considerable work in this area, relatively little is known about the RNases that actually carry out these processing reactions. In *Escherichia coli*, the tRNAs are co-transcribed as a single precursor molecule that is converted to the three mature tRNAs—23S, 16S, and 5S RNAs—in a multistep process (1, 2). The 5S RNA is released from the larger precursor RNA by the action of an endoribonuclease, RNase E (3). The product of this reaction is a pre-5S RNA molecule that contains 3 extra nucleotides at each end (4). The three 5' residues are thought to be removed by an exonucleolytic process (5), but no information is available on how the mature 3' end of 5S RNA is generated. In *Bacillus*, a single enzyme, termed RNase M5, is able to catalyze the maturation of both ends of 5S RNA in vitro (6, 7); however, as no mutants are yet available, it is not clear what role this enzyme plays in vivo.

Eight exoribonucleases that remove nucleotides in the 3' to 5' direction have been identified in *E. coli* (8, 9), and mutant strains lacking many of these enzymes, alone or in combination, are available (8, 10, 11). We have used these strains in combination with Northern blot analysis to address the question of whether any of these RNases might participate in the maturation of 5S RNA. Our results indicate that RNase T (12), an enzyme previously shown to participate in tRNA end turnover and tRNA processing (2), is required for maturation of the 3' terminus of 5S RNA. In the absence of RNase T, a 5S RNA molecule with 2 extra residues at its 3' end accumulates. Interestingly, cells containing this incompletely processed molecule and devoid of mature 5S RNA are nevertheless viable and grow only slightly slower than wild-type cells.

MATERIALS AND METHODS

**Bacterial Strains and Plasmids.** *E. coli* K-12 strain CA244 (*lacZ, trp, relA, spoT*) (11) was considered wild type for these studies. Exoribonuclease-deficient derivatives of CA244 were constructed by P1 transduction as described (10, 11, 13). The RNase T+, PH−, D−, and PNPase− (polynucleotide phosphorylase) strains all contain null mutations. The RNase II− and BN− mutations have not been characterized but lead to low RNase activity. Plasmid pKK3535 containing the *rmb* operon (14) was obtained from A. Dahlberg (Brown University).

**Materials.** [γ-32P]ATP was purchased from DuPont/New England Nuclear. T4 polynucleotide kinase and Moloney murine leukemia virus reverse transcriptase were from Gibco/BRL. RNasin was obtained from Promega. Sequence 2.0 was from United States Biochemical. *E. coli* RNase T was purified to homogeneity as described (15). All other chemicals were reagent grade.

**RNA Preparation.** Cells were grown in YT medium to *A* 550 ~ 1. Total RNA was isolated by phenol extraction as described (16). The RNA was used for analysis without further fractionation. tRNA was isolated from the ribosome fraction of cell extracts prepared as described (17). 5S RNA was purified from the total RNA of strain CA244 by gel filtration on Sephadex G-100.

**RNase T Treatment.** Total RNA, 5S RNA, or ribosomes were treated with purified RNase T in 20 mM glycine-NaOH, pH 8.9/10 mM MgCl2/5 mM dithiothreitol. Samples were incubated at 37°C for various times and diluted immediately into ice-cold gel loading buffer containing 25 mM Tris-HCl (pH 7.5), 0.5 mM EDTA (pH 8.0), 50% formamide, 0.1% xylene cyanol, and 0.1% bromphenol blue.

**Northern Blot Analysis.** RNA samples, dissolved in gel loading buffer, were loaded on a 5% polyacrylamide/8.3 M urea sequencing gel. The gel was run at 1700 V until the xylene cyanol dye migrated 31 cm. The RNA was transferred to a GeneScreenPlus membrane (DuPont) by electroblotting. The RNA was hybridized with a chemically synthesized 22-mer deoxynucleotide, GTTCGGCATGGGGTCAGGTGGG, complementary to residues 26–47 of 5S RNA, and labeled with 32P at its 5' end. The membrane was prehybridized at 42°C for 30 min in buffer containing 4× SSC, 0.5% SDS, 1× Denhardt's solution, and 0.1 mg of denatured salmon sperm DNA per ml. Hybridization was carried out at 42°C overnight in the same buffer containing 0.1% SDS and 32P-labeled probe. The membrane was washed twice for 15 min each with 4× SSC/0.1% SDS at 42°C prior to autoradiography.

**Primer Extension.** The protocol is based on the method described (18) with some modification. Thirty micrograms of

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Abbreviation: PNPase, polynucleotide phosphorylase.

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RNA was mixed with 0.5 pmol of $^{32}$P-labeled 5S oligonucleotide primer (same as used for Northern blotting) in 10 μl of buffer containing 10 mM Tris-HCl (pH 7.5), 300 mM NaCl, 2 mM EDTA (pH 8.0). The mixture was heated to 80°C for 4 min and then at 50°C for 2 h. Forty microliters of reverse transcriptase (400 units) in 1.25× first-strand buffer (as per the manufacturer’s protocol) and 0.5 μl of RNasin (15 units) were added to the hybridization mixture, and the primer was extended at 50°C for 30 min. The reaction was stopped by addition of 1 μl of 0.5 M EDTA (pH 8.0). DNA was precipitated with 1/10th vol of 3 M sodium acetate (pH 4.8) and 2 vol of cold ethanol. The pellet was recovered after centrifugation, washed with cold 75% ethanol, and dried. Samples were redissolved in 96% formamide containing 1 mM EDTA, xylene cyanol, and bromphenol blue. The products were separated on 6% polyacrylamide/8.3 M urea gels and detected by autoradiography as described above.

**Other Methods.** RNA was quantitated by $A_{260}$ measurement. DNA sequencing was carried out by the dideoxynucleotide method (19).

**RESULTS**

**Maturation of 5S RNA in RNase-Deficient Cells.** To determine whether the known *E. coli* exonucleases participate in 5S RNA processing, we performed Northern blot analysis of RNAs isolated from cells lacking one or several of these enzymes (Fig. 1). RNAs were probed with a DNA oligonucleotide complementary to residues 26–47, which are conserved among 5S RNAs from all seven *rrn* operons. Purified mature 5S RNA (lane 14), which is 120 nucleotides long, and a DNA sequence ladder were used as size markers. In wild-type cells (lanes 1 and 11), one predominant band is detected that is the same size as the 5S marker (lane 14). Thus, 5S RNA precursors are efficiently processed in wild-type cells. Likewise, cells lacking RNase PH (lane 2) or PNPass (lane 13) or RNase PH and PNPass (lane 12); RNAs II, D, and BN (lane 5); or RNases II, D, BN, and PH in combination (lane 6) all contain essentially only mature 5S RNA. In contrast, RNase T deficiency has a dramatic effect. In every cell in which RNase T is absent, either alone (lane 5), or in combination with other RNases (lanes 4 and 7–10), no mature 5S RNA is found. Rather, molecules larger than 5S RNA accumulate; the predominant product contains 2 extra residues, but a series of molecules with as many as 10 additional residues are observed. These data clearly demonstrate that RNase T is essential for the maturation of 5S RNA in *E. coli* and that in its absence incompletely processed 5S RNA molecules accumulate.

**5S RNA in Ribosomes of RNase T⁻ Cells.** To examine whether the defective 5S RNA present in RNase T⁻ cells actually assembles into ribosomes, RNAs from wild-type and RNase T⁻ cells were isolated and compared by Northern analysis. As shown in Fig. 2, 5S rRNA and total 5S RNA from wild-type cells have the mature size, as expected, whereas no mature 5S RNA is found in ribosomes from RNase T⁻ cells. As with total RNA, the predominant 5S RNA form in ribosomes is a molecule with 2 extra residues. Thus, the defective 5S RNA molecules are assembled into ribosomes and presumably, as the cells are viable, can function in protein synthesis.

**RNase T Action in Vitro.** To obtain additional evidence for direct involvement of RNase T in the maturation of 5S RNA, isolated RNA and ribosomes from the RNase T⁻ strain were incubated with homogeneous RNase T, and the products were analyzed by Northern blotting. With isolated RNA (Fig. 3A), the 5S RNA molecules with 2 extra residues (lane 2) are rapidly converted to the +1 form (lane 3), and, somewhat more slowly, two additional residues are removed to generate a −1 product (lanes 5–7). Although the −1 product is not observed in *vivo*, it is also generated when wild-type RNA (lane 8) is treated with RNase T under the same conditions (lane 9). When the same experiment is carried out with ribosomes (Fig. 3B), the 5S RNA from RNase T⁻ cells (lane 2) is rapidly converted to the mature size (lane 3) and remains at that size for up to 60 min (lanes 4 and 5). Likewise, 5S RNA in wild-type ribosomes is unaffected by RNase T (lanes 6–8), while isolated RNA is shortened by 1 residue (lanes 9 and 10). These data show that purified RNase T is able to remove the extra residues present on the 5S RNAs that accumulate in RNase T⁻ cells and that the removal is more rapid and more accurate with ribosomes than with isolated RNA. These findings indicate that the enzyme’s absence directly affects 5S RNA maturation.

**Analysis of the 5' End of 5S RNA.** Primer-extension analysis was carried out to determine the status of the 5' ends of 5S RNA molecules in wild-type cells and in cells lacking RNase T (Fig. 4). As shown in Fig. 1, RNAs from wild-type cells and RNase II⁻, D⁻, BN⁻, and PH⁻ cells contain mature 5S RNA, whereas those from an RNase T⁻ or from an RNase D⁻, BN⁻, PH⁻, and T⁻ cell accumulate the larger 5S molecules. Yet, based on the primer-extension analysis presented in Fig. 4, the 5S RNAs from all four strains have the same 5' termini. The major band in each lane, accounting for ~90% of the ends, corresponds to the mature 5' terminus of 5S RNA. A second,

**Fig. 1.** Northern blot analysis of 5S RNA from wild-type and exonuclease-deficient strains. RNA (1.0–1.5 μg per sample) was separated and analyzed as described. 5S RNA and a DNA sequencing ladder from a sequencing reaction of the RNase T gene were used as size markers. RNA from various strains was examined as follows: lane 1, wild type; lane 2, RNase PH⁻; lane 3, RNase T⁻; lane 4, RNases T⁻ and PH⁻; lane 5, RNases II⁻, D⁻, and BN⁻; lane 6, RNases II⁻, D⁻, BN⁻, and PH⁻; lane 7, RNases II⁻, D⁻, BN⁻, and T⁻; lane 8, RNases II⁻, BN⁻, T⁻, and PH⁻; lane 9, RNases D⁻, BN⁻, T⁻, and PH⁻; lane 10, RNases II⁻, D⁻, T⁻, and PH⁻; lane 11, wild type; lane 12, PNPass⁻, RNase PH⁻; lane 13, PNPass⁻; lane 14, 5S RNA standard; unlabeled lanes, DNA sequencing ladder.
mRNA; from sample) were isolated from RNase T− and T+ cells. Total RNA and rRNA (1.0–1.5 μg per sample) were separated on a 5% sequencing gel and Northern blotting was performed as described. Lane 1, wild-type total RNA; lane 2, wild-type rRNA; lane 3, RNase T− total RNA; lane 4, RNase T− rRNA. Positions of mature 5S RNA (M) and the +2 product are noted on the right.

mos much lighter band is 3 nucleotides longer and a small amount of the +1 band also is observed in each lane. These minor 5′ ends have been observed previously (5) and are also seen with isolated 5S RNA (data not shown). The experiment shown in Fig. 4 was carried out with limiting primer; however, identical results were obtained when the primer was present in excess (data not shown). These results demonstrate that the longer 5S RNA molecules that accumulate in RNase T− cells must have the extra residues at their 3′ ends, as expected from the 3′ → 5′ action of RNase T. Moreover, these data indicate that 5′ processing can take place independently of the reactions at the 3′ end of the 5S RNA molecule.

**DISCUSSION**

The data presented here demonstrate that (i) RNase T is essential for the 3′ maturation of 5S RNA precursors in *E. coli*; (ii) other known exoribonucleases play little or no role in this process; (iii) maturation of the 5′ terminus of 5S RNA proceeds independently of that at the 3′ end; and (iv) incompletely processed 5S RNA molecules are present in ribosomes and apparently function relatively normally. These findings, coupled with earlier studies that identified intermediates in processing of the 5′ terminus of 5S RNA in *E. coli* in vivo (5), indicate that both mature ends of 5S RNA in *E. coli* are generated by exoribonucleolytic trimming events. While these studies identify RNase T as the exoribonuclease acting at the 3′ terminus, the putative 5′ → 3′ exoribonuclease needed to remove the 3 extra 5′ residues remains to be found. Interestingly, maturation of the termini of *E. coli* 5S RNA is quite different from that suggested for *Bacillus*. In that case, a single endoribonuclease, RNase M5, is thought to generate both mature termini simultaneously by cleavage reactions (6, 7, 20). However, the *in vivo* role of that enzyme has not yet been proven.

RNase T was originally identified as the enzyme responsible for the end turnover of tRNA (12, 21). Subsequent work has shown that it also plays an important part in maturation of the 3′ end of tRNA (refs. 11 and 13; unpublished observations). It had been suspected that RNase T might be involved in processes other than tRNA metabolism because strains devoid of only RNase T activity grow 5–10 min slower than wild-type cells but are unaffected in tRNA maturation (11, 21). The studies presented here identify one other metabolic process requiring RNase T action. It remains to be seen whether the slowed growth of *mut* mutants is due to the incompletely matured 5S RNA molecules described here or whether RNase T has other essential functions as well. The features of RNA structure recognized by RNase T are presently unknown; however, the findings presented here suggest that tRNA precursors and 5S RNA precursors share a structural feature recognized by the enzyme.

An unexpected aspect of these studies is that some of the 5S RNA molecules that accumulate in RNase T− cells contain more than 3 extra 5′ residues and most contain 2 extra nucleotides. This is surprising because RNase E, the enzyme that generates the pre-SS RNA molecule, is thought to cleave at the +3 position (4). There are several possible explanations for these apparent discrepancies: (i) The earlier studies on RNase E cleavage specificity were carried out *in vitro* and with tRNA precursors derived from only a few of the seven *rnr* operons (3, 4, 22). The studies presented here are based on the accumulation of 5S RNA products *in vivo* from all the operons and thus may differ substantially from the earlier work. (ii) Other endoribonucleases with cleavage specificities different from RNase E may act during 5S RNA processing *in vivo*. (iii) The pre-5S RNA molecules that accumulate in RNase T− cells may have been altered. For example, polyadenylation of some molecules would extend their length and might explain...
Fig. 4. Primer-extension analysis of SS RNA from RNase T¹ and T⁻ cells. A 3²P-labeled, SS RNA-specific oligonucleotide was hybridized to RNA (30 µg) and extended by reverse transcriptase as described. A DNA sequencing ladder was prepared with the same 3²P-labeled primer and the cloned SS gene from pKK3535 and run side by side with the samples to determine sizes of the products (data not shown). The faint bands shorter than mature SS RNA are presumably prematurely terminated extension products. RNAs from the following strains are used: lane 1, wild type; lane 2, RNase T⁻; lane 3, RNase T⁺, D⁻, BN⁻, and PH⁺; lane 4, RNase D⁻, BN⁻, PH⁺, and II⁻; lane 5, primer.

the longer products, while 3' terminal trimming of the accumulated pre-SS RNAs could account for the presence of a +2 rather than a +3 product. Further studies will be needed to explain the difference between what actually accumulates in vivo and the earlier results reported with purified RNase E.

The difference in action of purified RNase T on the naked 5S RNA precursor and on the same molecule within the ribosome is of considerable interest. The ribosomal structure apparently alters the conformation of the 5S RNA precursor to allow more rapid and accurate processing than is possible with an isolated RNA molecule. Similar observations have been made by others for various rRNAs with extracts or partially purified enzyme preparations (23–26), and this seems to be a common theme for rRNA maturation.

The finding that ribosomes remain largely functional with incompletely processed 5S RNA molecules is now the second example of such a phenomenon. Earlier studies with cells affected in RNase III showed that the presence in ribosomes of certain 23S rRNA precursors is still compatible with cell survival (1). Clearly, ribosomes can maintain significant function with RNA molecules that are incompletely matured. This, of course, raises the very important question of why RNA molecules undergo a maturation process at all! Characterization of RNA processing pathways and identification of the enzymes responsible for processing events should bring us closer to answering this question.

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