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RNase III cleavage is obligate for maturation but not for function of *Escherichia coli* pre-23S rRNA

(rRNA processing/ribosomes/nuclease S1 mapping)

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ABSTRACT **RNase III makes the initial cleavages that excise *Escherichia coli* precursor 16S and 23S rRNA from a single large primary transcript. In mutants deficient in RNase III, no species cleaved by RNase III are detected and the processing of 23S rRNA precursors to form mature 23S rRNA fails entirely. Instead, 50S ribosomes are formed with rRNAs up to several hundred nucleotides longer than mature 23S rRNA. Unexpectedly, these aberrant subunits function well enough to participate in protein synthesis and permit cell growth. Consistent with the inference that RNase III cleavages are absolutely required for 23S rRNA maturation, when 50S ribosomes from a strain deficient in RNase III were incubated with a ribosome-free extract from a RNase III$^+$ strain, rRNA species processed by RNase III and species with normal mature 23S rRNA termini were produced.**

rRNA in *Escherichia coli* is synthesized as one transcript containing 16S, 23S, and 5S rRNA as well as precursor sequences. This transcript is thought to be processed first by RNase III, which cleaves the precursor at double-stranded stems and separates the three rRNA species from one another before transcription is complete; other processing activities then remove the remaining precursor sequences during ribosome formation, generating the mature termini (1, 2). Because RNase III initiates processing, and processing has been generally considered indispensable to produce functional RNA molecules, it was unexpected that mutants deficient in the enzyme could survive. It was originally suggested that a low level of RNase III might remain in such mutants or that other RNases might form an alternative pathway to yield mature rRNA (3–5). These ideas have persisted in recent discussions, but both are disproven here in the case of 23S rRNA formation. Nuclease S1 mapping is used to show that the lesion in RNase III is complete in these mutants, but no backup pathway replaces the function of RNase III. The 16S rRNA is matured normally without any preliminary RNase III cleavage, but 23S rRNA exists only as unmatured species. Maturation of 23S rRNA is therefore absolutely dependent on RNase III action, but at least some of the unmatured 23S rRNA species detected must function in protein synthesis because these mutant strains are viable. The maturation of the unprocessed 23S rRNA in 50S ribosomes is shown to occur in subcellular extracts of a RNase III$^+$ strain.

MATERIALS AND METHODS

The termini of rRNA species in two RNase III-deficient strains, ABL1 and AB301-105 (6, 7), were assessed by their capacity to hybridize to DNA probes composed of complementary sequences of rDNA. ABL1 and AB301-105 cells were grown in Luria broth at 30°C to an optical density of 0.25 at 550 nm and then harvested over ice. Total RNA was prepared by extracting cells directly at 65°C with phenol in the presence of 1% sodium dodecyl sulfate. The RNA was precipitated once with LiCl (8) and twice with ethanol and was then suspended at a standard concentration. Single-stranded DNA hybridization probes for each terminus of 16S and 23S rRNA were prepared from subclones of pKK 3535 [a pBR322 derivative containing one of the seven ribosomal DNA operons from *E. coli* (*rrnB*); see ref. 9] by restriction enzyme digestion, end-labeling, and strand separation (see legend to Fig. 1 and ref. 10). Probes contained 30–100 nucleotides of mature rRNA sequence and at least 90 nucleotides of precursor rRNA sequence. After RNA·DNA hybridization at 65°C for 2 hr in DNA excess, samples were chilled, quenched with 10 vol of cold nuclease S1 buffer (11, 12), and digested with nuclease S1 (3,000 units/ml) (Sigma) for 30 min at 30°C. Samples were processed by phenol/chloroform extraction and repeated ethanol precipitation. The RNA in some samples was hydrolyzed before electrophoresis (13). The protected DNA was analyzed by electrophoresis in 20% or 6% polyacrylamide sequencing gels as described by Maxam and Gilbert (10).

For the preparation of soluble proteins, *E. coli* strains D10 and ABL1 were grown in Luria broth to an optical density of 0.6 at 650 nm, harvested on ice, ground with alumina, and centrifuged in 10 mM Tris·HCl, pH 7.4/5 mM $MgCl_2$/2 mM $CaCl_2$ at 12,000 × *g* for 20 min. The supernatants were brought to 1 M NH_4Cl and 30 mM $MgCl_2$ and centrifuged onto a layer of 60% sucrose (1 ml for every 5 ml of supernatant) in the same buffer, at 100,000 × *g* for 3.5 hr. The supernatant was taken as total soluble protein ("S100 fraction"). This fraction still contained some rRNA that reacted with the DNA hybridization probe. In a subsequent sucrose gradient centrifugation, the processing activity moved more slowly than the rRNA yielding fraction 1 depleted of rRNA (see Fig. 3).

Ribosomes from the two strains were obtained as a pellet after centrifugation of the crude extracts of strains ABL1 and D10 to prepare S100 fraction as above. The respective ribosome pellets were suspended in 10 mM Tris·HCl, pH 7.4/0.2 mM $MgCl_2$ and centrifuged at 5°C in a linear gradient of 10–30% sucrose in the same buffer at 80,000 × *g* for 15 hr. The 50S and 30S ribosomal subunits were precipitated from the gradient fractions with 2 vol of ethanol. The ribosomal subunits were suspended in 10 mM Tris·HCl, pH 7.4/5 mM $MgCl_2$ and stored frozen until used.

To test for processing of the 5′ end of 23S RNA in 50S subunits, 5 μg of 50S subunit and 2.5 μg of 30S subunit were incubated in 50 μl of an *in vitro* protein synthesis system (as described in ref. 13, but without any added DNA or messenger RNA). Incubation was at 37°C for 15 min, with 20 μg of S100 fraction protein or 2–8 μg of fraction 1 protein. Total RNA was then phenol-extracted from each of the reaction mixtures and hybridized with an excess of the DNA probe corresponding to the 5′-terminus of 23S RNA, and then the samples were processed for nuclease S1 mapping. The

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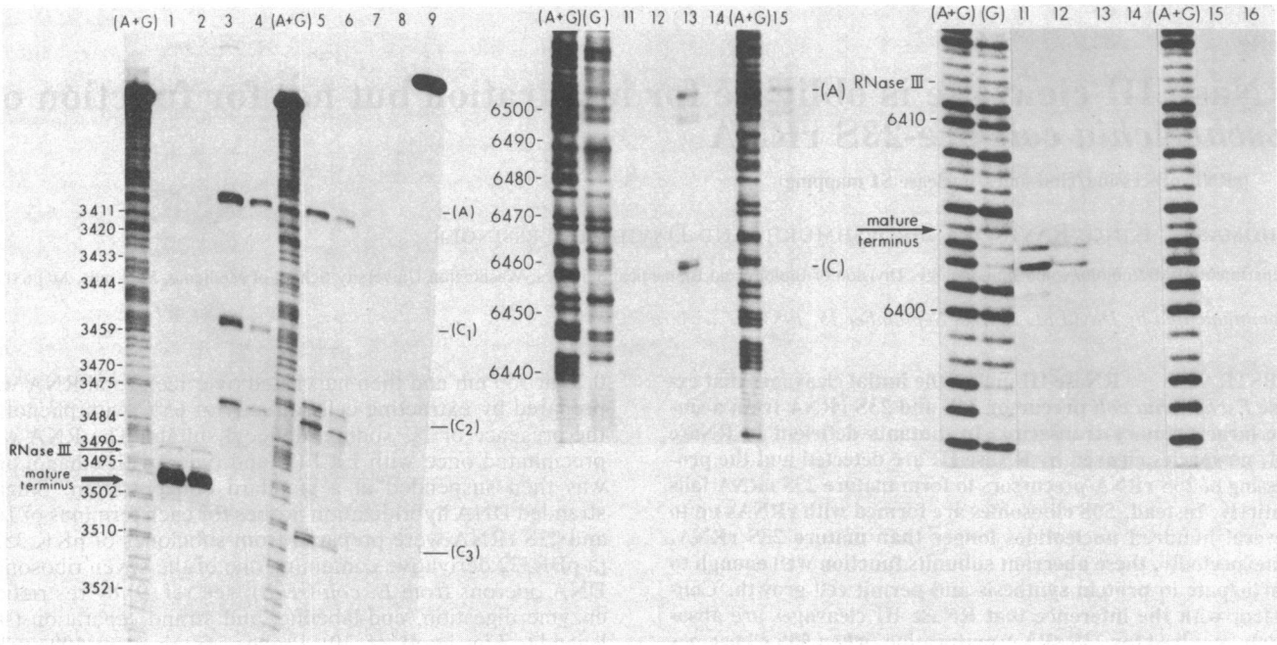

FIG. 1. Sequence analysis. (*Left*) Six percent sequence analysis gel for the 5′ end of 23S rRNA is shown. An end-labeled, single-stranded DNA probe [complementary to 107 nucleotides of mature 23S rRNA sequence at the 5′ end and 95 nucleotides of adjacent precursor rRNA sequence; nucleotides 3,404–3,606 of the *rrnB* sequence (9) with 129 nucleotides of pBR322 sequence at its 3′ end] was either treated for Maxam and Gilbert guanine (G) and guanine + adenine (G+A) sequence reactions (10) or hybridized to 0.118 μg of either mature 23S rRNA from strain D10 (lanes 1 and 2), total AB301-105 RNA (lanes 3 and 4), total ABL1 RNA (lanes 5 and 6), or yeast RNA (lanes 7 and 8) and then treated with nuclease S1. The samples in lanes 2, 4, 6, and 8 were treated with alkali before electrophoresis (13). In lane 9, the untreated nuclease S1 probe is shown. The sequence reactions are numbered according to Brosius *et al.* (9) and the positions of the normal mature 5′ terminus of 23S rRNA and the RNase III cleavage site are indicated. The species identified in the RNase III-deficient strains are also indicated (A, C_1, C_2, and C_3). (*Middle* and *Right*) A similar experiment for the 3′ end of 23S rRNA is shown. The nuclease S1 probe used was complementary to 29 nucleotides of mature 23S rRNA sequence at the 3′ end and 94 nucleotides of precursor rRNA sequence [nucleotides 6,374–6,497 of the *rrnB* sequence (9) with 104 nucleotides of pBR322 sequence at its 5′ end]. Nuclease S1 reactions were carried out with 0.118 μg of either mature 23S rRNA (lanes 11 and 12), total AB301-105 RNA (lanes 13 and 14), or total ABL1 RNA (lanes 15 and 16). Samples in lanes 12, 14, and 16 were treated with alkali before electrophoresis (13) (a portion of the sample for lane 14 was lost during preparation). Portions of these samples, along with the same DNA probe treated for Maxam and Gilbert sequence reactions (10), were electrophoresed both in a 6% sequence gel (*Middle*) and in a 20% sequence gel (*Right*) to allow good resolution in the size range of interest. The DNA sequence lanes are numbered according to Brosius *et al.* (9). The novel species (A and C) and the normal 23S rRNA 3′ terminus and the RNase III site are indicated.

DNA·RNA hybrids were extracted with phenol and analyzed, after denaturation in formamide, by electrophoresis in 10% acrylamide gels (acrylamide/bisacrylamide, 30:1) containing 8.3 M urea, with 200 mM Tris·borate/4 mM EDTA, pH 8.4, as running buffer.

RESULTS

All four termini of the "16S" and "23S" rRNA species of the two RNase III-deficient strains (ABL1 and AB301-105) were examined using appropriate DNA hybridization probes. Both total cellular RNA and rRNA extracted from 70S ribosomes were investigated, and each gave similar results. The termini of the standard *E. coli* strain D10 (14) were also examined as a control. As expected, 80–90% of both 16S and 23S species in strain D10 had mature termini, with the bulk of the remaining rRNA termini corresponding to RNase III-cleaved intermediates.

No species arising from RNase III cleavage were detected in ABL1 or AB301-105, suggesting that the RNase III lesion in these strains is complete. The majority (95%) of 16S species detected in either strain had mature 5′ and 3′ termini (unpublished observations; see ref. 15). However, no rRNA species in ABL1 or AB301-105 have the mature 5′ or 3′ 23S rRNA termini found in D10 (Fig. 1, lanes 3–6 and 13–16). Instead, other termini, some longer and one shorter than normal 23S rRNA, were found. Four putative termini were identified at the 5′ end and two at the 3′ end of 23S rRNA. The gel bands corresponding to these termini (A, C_1, C_2, and C_3 at the 5′ end; A and C at the 3′ end) are indicated in Fig. 1 and their positions relative to the sequence of the *rrnB* rDNA operon are shown in Fig. 2. (The relative amounts of these species present were the same in two independent rRNA preparations.) Fig. 2 represents the precursor sequences surrounding 23S rRNA in the secondary structure suggested by several investigators (9, 16).

These results suggest that cleavage by RNase III, which occurs first kinetically during 23S rRNA production, is also mechanistically first. As an initial test of this idea, we extended the studies of processing of 23S pre-rRNA to subcellular systems. 50S ribosomes containing 23S rRNA with extra sequences at each end were isolated from strain ABL1 and incubated with soluble proteins from a wild-type strain. Both termini generated by RNase III cleavage and normal mature 23S rRNA 5′ termini were produced under these conditions (Fig. 3, lanes 5–8). In contrast, incubation with extracts from RNase III-deficient strains yielded no further processing (lane 4). These results are again consistent with an obligatory initial cleavage of 23S rRNA precursors by RNase III.

DISCUSSION

The processing reactions that occur in wild-type and RNase III-deficient strains are summarized schematically in Fig. 4.

The abnormal state of 23S rRNA in RNase III-deficient strains probably escaped detection in earlier studies because of the large size of 23S rRNA (2,904 nucleotides). The difficulties of mapping such a large molecule are considerable, and even an extra sequence of 100 nucleotides would pro-

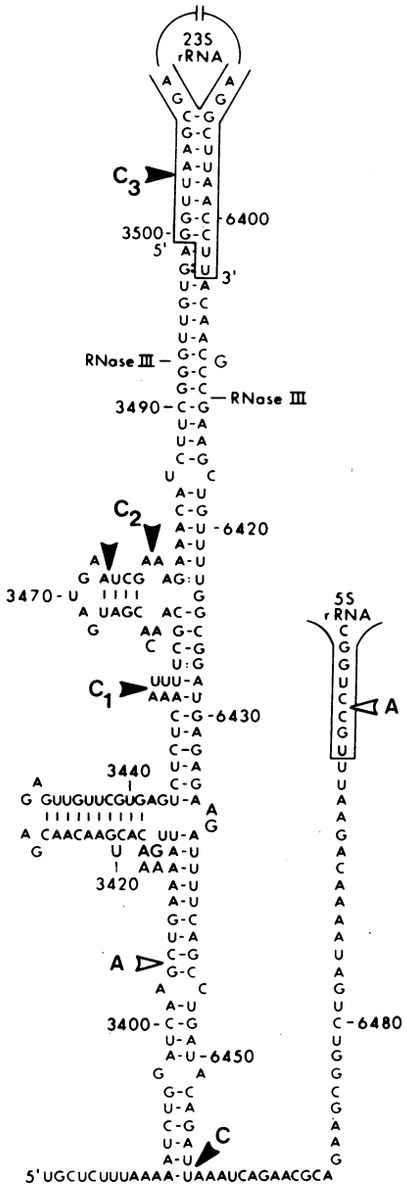

FIG. 2. The nucleotide sequences adjoining the 5′ and 3′ ends of 23S rRNA are shown in the secondary structure suggested in refs. 9 and 16. Positions labeled with open arrowheads (A) correspond to protection of the full length of *rrnB* sequence contained in a nuclease S1 probe used for Fig. 1. All other cleavage sites detected in Fig. 1 are labeled with closed arrowheads (C_1, C_2, and C_3 at the 5′ end and C at the 3′ end).

duce a size change of less than 4%. Such differences would probably not have been detected in the gel electrophoresis conditions used in previous studies (17).

Double-stranded RNA stems with intact RNase III sites have previously been isolated as such from these strains by ribonuclease treatment of cellular RNA (6, 18). Those results are entirely consistent with the nuclease S1 results presented here, but they were not quantitative and did not suggest whether a sizeable fraction of 23S termini remain unmatured in these strains.

Since the longest species observed at both the 5′ and 3′ ends of 23S rRNA correspond to protection of the full *rrnB* sequences contained in the nuclease S1 probes, the true termini of these species are probably further from the normal mature termini of 23S rRNA than is shown in Fig. 2 (extending toward processing sites for tRNA at the 5′ end and 5S

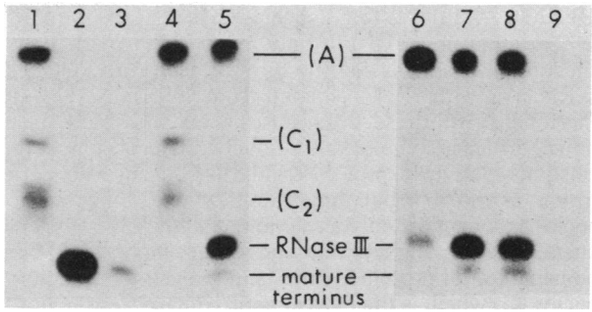

FIG. 3. Processing of 23S pre-rRNA in 50S ribosomes by soluble protein preparations. 50S ribosomes from *E. coli* strains D10 or ABL1 were incubated with or without soluble proteins, and total RNA from the reaction mixtures was extracted and hybridized with a labeled DNA probe corresponding to the 5′ end of 23S rRNA, and then the hybrid was digested with nuclease S1 and fractionated by gel electrophoresis. The positions of hybrids corresponding to species A (uncut pre-rRNA) and species C_1 and C_2 (Fig. 1), RNase III-cleaved RNA, and 23S rRNA with a normal mature terminus are indicated. Lanes: 1 and 2, 50S ribosomes from ABL1 and D10, respectively, incubated without any soluble protein fraction; 3, D10 S100 fraction incubated without ribosomes; 4 and 5, 50S ribosomes of ABL1 incubated with S100 fraction from ABL and D10, respectively; 6–8, 50S ribosomes from ABL1 incubated with 2, 4, and 8 μg of fraction 1, respectively; 9, fraction 1 of D10 incubated without ribosomes.

rRNA at the 3′ end; see ref. 5). Some of the additional termini observed could be formed *in vivo* by nonspecific nucleolytic attack in regions that remain single-stranded in the RNA secondary structure (as in Fig. 2).

Densitometry of autoradiograms revealed that the relative amounts of these novel termini differ somewhat between

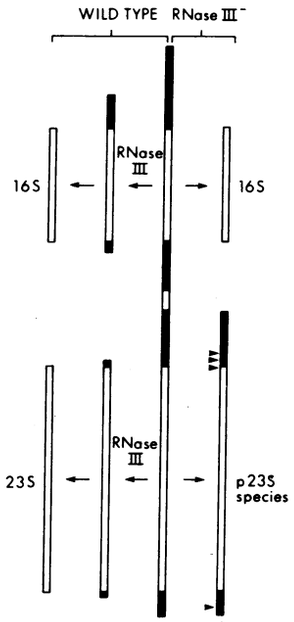

FIG. 4. Processing of rRNA in wild-type and RNase III-deficient *E. coli*. A full pre-rRNA transcript is shown schematically in the center, with its 5′ end at the top. Precursor sequences are in black; mature sequences are in white and are not drawn to scale. Arrows pointing left show the processing steps in a RNase III^+ strain. RNase III produces intermediate species that are further processed to form mature 16S and 23S rRNA. Arrows pointing right indicate the processing steps in RNase III^- strains. The 16S rRNA is produced directly without the formation of intermediates by RNase III. Mature 23S rRNA is not formed, but several unmatured 23S rRNA species (indicated by arrowheads) are produced.

ABL1 and AB301-105 (e.g., species C_3 accounted for 25% of the 5′ termini in ABL1 but only 6% in AB301-105; unpublished data). A similar distribution was seen with total cellular RNA or with rRNA isolated from 70S ribosomes. The functionality of these different species, however, is unknown. At least some of these novel 23S species must form ribosomes active in protein synthesis, because no normal termini are present but the strain is viable. Since any one species accounts for only a fraction of all the 23S rRNA molecules, it seems unlikely that one species alone could be responsible for all protein synthesis in the cells. For example, species C_3, which is the closest approximation to the normal mature 5′ terminus in either strain (four nucleotides shorter than the usual mature 23S 5′ terminus), accounts for only 6% of the termini in AB301-105, and it seems very unlikely that the strain could survive with only 6% of its 50S ribosomes functioning in protein synthesis. These questions could be addressed directly by measuring *in vitro* protein synthesis using 50S ribosomes from these RNase III-deficient strains, by analyzing the termini of 50S subunits from *in vivo* polysomes, or by reconstituting these novel 23S rRNA species with r-proteins *in vitro* and assaying their protein-synthesizing capacity before and after *in vitro* processing.

Because RNase III cleavage usually occurs very early during ribosome formation, the cleavage sites and mature termini of 23S rRNA could become inaccessible when complete 50S ribosomes have formed with pre-23S rRNA. For example, a double-stranded stem at the base of a loop of 23S pre-rRNA (the recognition site for RNase III) could be disrupted or buried in the body of the assembled 50S ribosome. However, the results in Fig. 3 show that at least the 5′ cleavage site remains accessible in the appropriate conditions even in intact ribosomes. There are at least two practical consequences of these results: first, assuming that the structure shown in Fig. 2 exists *in vivo* [as suggested in several models for the secondary structure of 23S rRNA (9, 16)], the 5′ and 3′ termini of 23S rRNA can be hydrogen-bonded even in functional 50S ribosomes. Very likely, such a structure would extend out from the ribosome at the point at which the 3′ terminus of 23S rRNA has been localized (19, 20). Second, 50S ribosomes from strain ABL1 provide a substrate for the purification of the processing activity or activities that generate the mature termini of 23S rRNA. Using nuclease S1 as described here, the assay has no background (Fig. 3).

In vivo, the altered 50S subunits in these strains deficient in RNase III may result in some of their puzzling phenotypic characteristics. Both strains have decreased growth rates (≈30% slower than D10 in Luria broth at 30°C), and AB301-105 is partially temperature sensitive (5). These phenotypes are not due to a temperature-sensitive RNase III, because there are no RNase III cleavage products even at permissive temperatures. The chemical half-life of bulk mRNA and of tryptophan operon mRNA are also increased 2-fold (21); and some proteins, particularly β-galactosidase (21), are not made or are made in decreased amounts (22). Interestingly, extracts from these strains work poorly for *in vitro* protein synthesis, although all other nucleases tested are present at the same levels in RNase III^- and otherwise isogenic RNase III^+ strains. All of these features could result from the modified function of the unmatured rRNA in 50S ribosomes rather than from the failure of other RNase III-mediated reactions in the cells.

These results raise the general question of the extent to which precursors of structural RNAs can function in cells before processing is completed. Evidence concerning *E. coli* 16S rRNA is inconclusive. Precursors to 16S rRNA bind ribosomal proteins *in vitro*, but form inactive ribosomal particles (23). On the other hand, precursors to 16S rRNA have been found in polyribosomes isolated from whole cells (24, 25).

The unmatured 23S rRNA molecules described here thus provide a clear case of a structural precursor RNA that functions *in vivo*. Even in this case, further processing may well enhance the function of these 50S ribosomes. Perhaps, as suggested previously (26–28), rRNA species evolved with extra adjacent sequences to promote ribosome formation. The removal of the extra sequences is then not absolutely required for the function of at least some RNAs, but may represent a later evolutionary development that further improves ribosome activity.

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