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Processing pathway of *Escherichia coli* 16S precursor rRNA

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ABSTRACT

Immediate precursors of 16S rRNA are processed by endonucleolytic cleavage at both 5′ and 3′ mature termini, with the concomitant release of precursor fragments which are further metabolized by both exo- and endonucleases. In wild-type cells rapid cleavages by RNase III in precursor-specific sequences precede the subsequent formation of the mature ends; mature termini can, however, be formed directly from pre-16S rRNA with no intermediate species. The direct maturation is most evident in a strain deficient in RNase III, and the results in whole cells are consistent with results from maturation reactions in vitro. Thus, maturation does not require cleavages within the double-stranded stems that enclose mature rRNA sequences in the pre-16S rRNA.

INTRODUCTION

In procaryotic cells, ribosomal DNA is transcribed into 30S RNA chains. These include 16S, 23S, 5S rRNA and tRNAs as well as extra leader, spacer and trailer sequences. Processing of the long primary transcripts to form mature rRNA involves cleavages and trimming by a number of enzymes (1). In E. coli cells, processing is rapid, and most rRNA is mature. Only 1-2% of rRNA is in the form of very long precursors, and about 10-20% is in precursors already rapidly processed by RNase III (2), which cuts at specific sites in the transcript to form direct precursors of rRNA [pre-16S, pre-23S, and pre-5S rRNAs]. Other enzymes are then involved in the final maturation of the pre-rRNAs.

The 16S and 23S rRNA sequences in the primary transcript are each enclosed by a double-stranded stem formed from complementary sequences flanking the 5′ and 3′ termini of the mature species. The major RNase III cleavages occur at staggered positions in each double-stranded stem. The pathway of subsequent processing has

been studied in detail for 23S pre-RNA (3); but the analysis of 16S pre-rRNA maturation has remained less complete. The most suggestive results have come from studies of cells deficient in RNase III. Such cells contain no RNase III-cleaved species; but 16S molecules form with normal mature termini at the same rate as in the wild-type strain. These results have implied that enzymes involved in final maturation may function whether or not RNase III has acted (2).

Such enzymatic activities involved in the maturation of 16S rRNA termini have been partially purified (4,5), and a mutant with altered maturation at the 5′ end has been partially characterized (5). Earlier technology, however, often did not discriminate whether correct termini were produced during maturation, or the extent to which maturation was endonucleolytic. It was also unclear whether the enzymes that act at the 5′ and 3′ termini are distinct, or as some have suggested, may be identical (reviewed in ref.6).

In analogy to earlier studies with 23S rRNA (2,3,7,8,9), S1 nuclease protection assays can determine the intermediates that form during the maturation of pre-16S rRNA. In particular, one can distinguish whether long pre-rRNA molecules are directly cleaved endonucleolytically at mature rRNA termini, or whether cleavages occur first at a distance from a terminus, and are followed by further exonucleolytic action. Inferences of the pathway in wild-type *E. coli* can then be extended by comparing the results with those in an RNase III-deficient strain.

Maturation of pre-rRNA is difficult to follow in the presence of the high levels of mature 16S rRNA in cells. It is nevertheless possible to assess processing by detecting precursor-specific fragments released during maturation (10). We have utilized two approaches: 1. looking for fragments released during processing reactions in cell extracts (i.e., *in vitro* maturation reactions); and 2. detection of precursor fragments in steady state cellular RNA (i.e., *in vivo* maturation reactions).

MATERIALS AND METHODS

Bacterial strains. *Escherichia coli* strain D10 (wild-type, ref.11) was grown at 37°C in Luria broth to an optical density of

0.55-0.65 at 550 nm. RNase III-deficient strain ABL1 (12) was grown in broth at 30°C. The cells were harvested on ice, washed with ice-cold buffer (10 mM Tris-HCl, pH 7.4/ 5 mM $MgCl_2$/ 2 mM $CaCl_2$) and stored at -70°C.

Preparation of total RNA, ribosomes, and rRNA. Cells were grown as described above and harvested at two different concentrations, at an optical density of 0.35 or 0.70 at 550 nm. Total RNA was extracted as described (13). Ribosomes were prepared according to methods in ref.2. RNA was isolated from ribosomes by phenol extraction as described (13).

Preparation of S100 and ribosome wash. S100 and "ribosome wash" fractions (made with 0.2 M or 1 M salt) from strain ABL1 and D10 were as described (13).

Maturation reaction in vitro. 5 μg of ribosomes from the RNase III-deficient strain ABL1 was incubated in a total volume of 50 μl in 25 mM Tris-HCl, pH 7.8/ 100 mM NH_4Cl/ 10 mM $MgCl_2$/ 6 mM 2-mercaptoethanol, with or without 5 μl of ribosome wash fraction at 37°C for 30 min. After the reaction, RNA was extracted, precipitated with ethanol, and dissolved in hybridization buffer.

S1 nuclease mapping. Probes and their source have been described in detail (13). The single-stranded probe for the 5′ mature end extends from nucleotide 1353 to 1540 (14) and contains 23 nucleotides of mature rRNA and 165 nucleotides of precursor sequence (Fig. 1, bottom). For the study of 3′ termini, the probe DNA was complementary to nucleotide 2959 to 3156, including 98 nucleotides of precursor and 100 nucleotide of mature sequence. [It also contains 143 nucleotides of pBR322 sequence distal to the mature 16S rRNA region (Fig. 3, bottom).]

5′-end labeled probe was prepared by treatment of DNA with bacterial alkaline phosphatase followed by labeling with [γ-^{32}P] ATP and T4 polynucleotide kinase (15). The 3′ end of the probe was labeled with the Klenow fragment of DNA polymerase I and appropriate [α-^{32}P] deoxynucleoside triphosphates (15). Hybridization of end-labeled single-stranded DNA fragment (4,000-6,000 cpm) and total RNA (5 μg) or rRNA (2.5 μg) in hybridization buffer (100 mM HEPES, pH 6.4/ 400 mM NaCl/ 1 mM EDTA) was carried out as described (13) at 63°C for 3 h. The reaction was terminated by rapid 10-fold dilution with 200 μl of

digestion buffer (280 mM NaCl/ 50 mM sodium acetate, pH 4.6/ 4.5 mM zinc sulfate) containing 40 units of S1 endonuclease. After incubation for 1 h at 37°C, S1 resistant nucleic acids were precipitated with 2.5 vol of ethanol in the presence of 10 μg of yeast tRNA at -45°C for 30 min. The nucleic acids were redissolved in 10 μl of 90% formamide, denatured by boiling for 2 min, and fractionated in an 8% polyacrylamide gel (30:1 acrylamide:bisacrylamide) containing 8.3 M urea/ 90 mM Tris-borate/ 2 mM EDTA, pH 8.0.

RESULTS

Cleavages at the 5' end of pre-16S rRNA: processing in cell extracts.

As a first test for possible cleavages of pre-16S rRNA incubated in the absence of RNase III, ribosomes from the RNase III-deficient strain ABL1 were treated in buffered salt solution with various soluble protein preparations (see Methods and legend to Fig. 1), and the termini produced were assessed. RNA was extracted from untreated or treated ribosomes, and protection from S1 nuclease was attempted with a single-stranded DNA probe complementary to a portion of the mature 16S rRNA sequence and adjacent precursor sequences. The probe covers 23 nucleotides of mature and 165 nucleotides of adjacent precursor sequence (Fig. 1, bottom), and was labeled at the end in the precursor region. Such a probe allows the detection of termini present before or produced during the the processing reaction (Fig. 1).

The steady state levels of termini were first compared in RNA isolated from ribosomes of strain ABL1 (Fig. 1, lane 1) and the wild-type strain D10 (lane 6). The RNAs from both strains show a species P, fully protected from S1 nuclease action, arising from very long primary transcripts. Both the strains also contain a species M. Comparing its size with the sequence of *rrnB* DNA (14), it corresponds to the fragment expected from direct cleavage at or very near the 5'mature terminus. [In other experiments (data not shown), the fragment size was verified +/- 3 nucleotides by comparison to a sequencing ladder made from the probe, as in ref. 2.] The presence of such a fragment indicates that a single

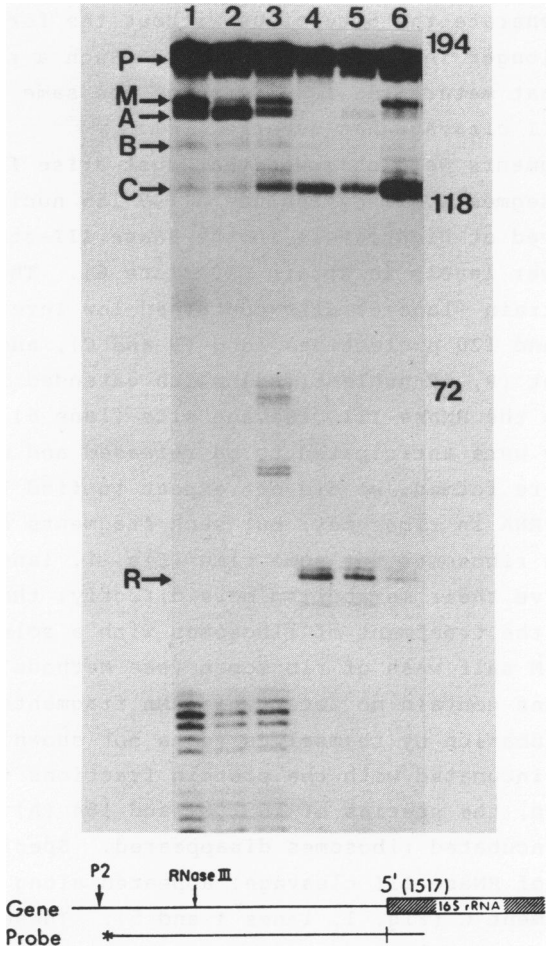

Figure 1. S1 nuclease mapping analysis of products of processing during maturation reaction at 5' mature end in vitro. Ribosomes from strain ABL1 were treated with different preparations of cell extract (see Materials and Methods). RNA was extracted from untreated and treated ribosomes (see Methods) and was separately hybridized with a single-stranded probe 188 nucleotides long (diagrammed at the bottom). Subsequent S1 nuclease treatment and analyses of products on 8% urea-polyacrylamide denaturing gels as described in Material and Methods. Lanes 1 and 6, untreated rRNA of strains ABL1 and D10, respectively. Lanes 2 and 3, after treatment with S100 or 1M ribosome salt wash from strain ABL1. Lanes 4 and 5, after treatment with 0.2 M ribosome salt wash or 1M ribosome salt wash from the RNase III-positive strain D10.

cleavage can generate the 5'terminus without the formation of any substantially longer intermediate species. Such a result extends the findings that mature 16S rRNA forms at the same rate whether or not RNase III cleavage has occurred (2).

Other fragments were observed that must arise from metabolism of spacer RNA segments. They include a 149-155 nucleotide RNA (A) that was observed at high levels in the RNase III-strain (lane 1) and at much lower levels in strain D10 (lane 6). The RNase III-positive strain (lane 6) also contained low levels of fragments 138 and 120 nucleotides long (B and C), and a still smaller fragment (R, 50 nucleotides) which extended from the end of the probe to the RNase III cleavage site (lane 6).

Since they were anticipated to be released and degraded as soon as they were formed, we did not expect to find fragments of cleaved spacer RNA in ribosomes; but such fragments seem to remain associated with ribosomes for some time (Fig. 1, lanes 1 and 6). To try to observe their metabolism more directly, they were assessed after the treatment of ribosomes with a soluble protein fraction or a 1M salt wash of ribosomes (see Methods). The protein fractions contain no detectable RNA fragments, and produce none during incubation by themselves (data not shown). But when ribosomes were incubated with the protein fractions from the wild-type strain, the species of 165 (M) and 154 (A) nucleotides observed in unincubated ribosomes disappeared. Species (R), characteristic of RNase III cleavage, appeared along with a 120 nucleotide fragment C (Fig. 1, lanes 4 and 5). These results suggest most simply that longer fragments, including (M), are degraded to others of the intermediates seen in cellular RNA (Fig. 1, lane 6) by enzymes present in the ribosome wash.

Protein fractions from strain ABL1 were consistently less efficient in the maturation reaction in vitro. Treatment with S100 fraction (Fig. 1, lane 2) or ribosomal wash (lane 3) led to increased levels of fragment A or C, respectively. [Additional shorter products of RNA metabolism, 34 and 67-70 nucleotides long, (not indicated) were also observed.]

Because the released fragments were so unstable, it was impossible to detect net formation of the 5'terminus in vitro; but

oligonucleotides arising from the precursor sequence abutting the mature terminus have been previously reported, produced with partially purified fractions as the source of enzyme (4). The detected amounts of fragment M and metabolic products depend both on the extent of the reaction and the rate of degradation of the fragments produced; but for the inferences made here, only the production of particular fragments, and not their levels, is scored (see Discussion).

Cleavages at the 5′ terminus in vivo.

Fig. 2 (left) shows the RNA termini present in total RNA extracted from strains ABL1 (lanes 1 and 2) and D10 (lanes 3 and 4), as detected by the S1 nuclease protection probe used in Fig. 1. The termini are very similar to those found in isolated ribosomes (Fig. 1), though the amounts vary somewhat between strains and preparations. For example, fragment (M) occurs at relatively low levels in strain D10, probably because of its rapid degradation to fragments B, C, and smaller bits. Similarly, the RNase III-cleaved fragment had been degraded to smaller bits undetectable in total RNA preparations under the hybridization conditions used. Also, fragment B is present in appreciable amounts in the wild-type strain, but is at very low or neglegible levels in preparations from untreated ribosomes (Fig. 1, lane 6) or from ribosomes after the processing reaction in vitro (Fig. 1, lanes 4 and 5).

The interpretation that cleavage at site R has indeed occurred, and that one product is differentially unstable, is strongly supported by the detection of the other product of the cleavage reaction. It is seen with the same probe used in Fig. 2 (left) labeled at its opposite end -- at the mature terminus ("b", Fig. 2, bottom). Results are presented in Fig. 2 (right). The 23 nucleotide fragment which would extend from the labeled end of the probe to the 5′ end of mature 16S rRNA was faint in the hybridization conditions used, and was only clearly seen at lower stringency. More importantly, the probe strongly detected both the fully protected precursor (P) and one major fragment (R′), the species generated by RNase III, in total cellular RNA from strain D10 (lanes 8 and 9). The R′ fragment abuts the R cleavage site

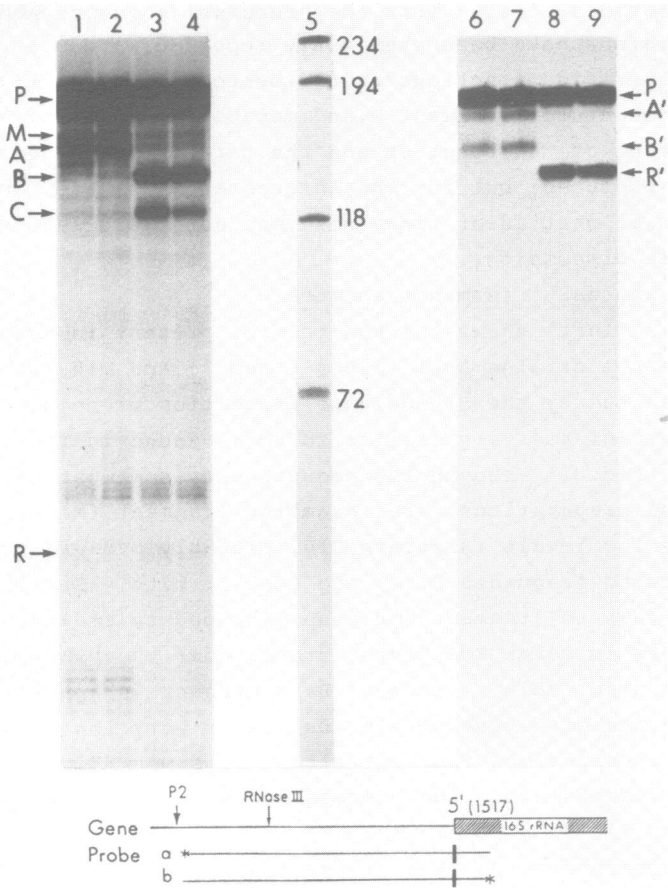

Figure 2. S1 nuclease mapping of 5′ termini in 16S rRNA species in strain ABL1 and D10. Total RNA (5 μg) from the RNase III-deficient strain ABL1 or wild-type D10 analyzed with probe (diagrammed at bottom) as in Fig. 1. Lanes 1-4 (left) with probe labeled at the end within the precursor sequence (bottom, a); lanes 6-9 (right), same probe labeled in the mature sequence (bottom, b). Lanes 1,6 and 2,7 are total RNA from strain ABL1 at two different stages of cell growth (see Materials and Methods). Lanes 3,8 and 4,9 corresponding to 1,6 and 2,7, but with RNA from strain D10. Lane 5, Marker. Asterisk: the labeled end of the probe. P,M,A,B,C and R label various products of RNA metabolism (see text).

and extends from it into mature rRNA sequence; i.e., the probe detected the termini present on rRNA rather than on detached fragments of precursor sequence.

The fragment arising from cleavage at R was, as expected, not

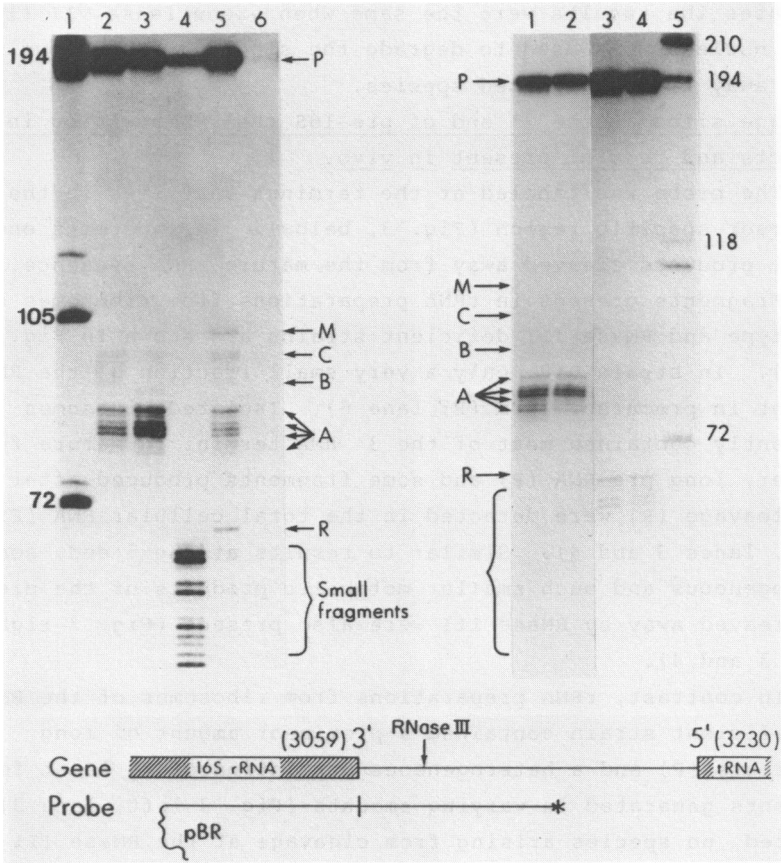

Figure 3. S1 nuclease mapping of (i) products of processing during maturation reaction at 3' mature end in vitro (left) and (ii) 3' termini in 16S rRNA species in strain ABL1 and D10 (right). Treatment and analyses as described in legend to Fig. 1. The probe is diagrammed at the bottom and described in Materials and Methods. Left, lanes 2 and 6 are rRNA from strain ABL1 and D10 respectively. After treatment with 1 M ribosome salt wash from ABL1, lane 3; from D10, lane 4. Lane 5, treatment with mixture of 1 M ribosome salt wash from D10 and S100 preparation from ABL1. Lane 1, marker. Right, lanes 1, 2 and 3, 4 are RNA from strain ABL1 and D10, respectively, at two stages of cell growth. Lane 5, Marker.

present in strain ABL1 (Fig. 2 lanes 6 and 7). Rather, the major species observed included the fully protected precursor and two other species (A' and B') representing mature RNA with 147-157 and 131 extra nucleotides. The longer species, which were predominant in ABL1, were relatively rare in strain D10 (see Discussion). In

all cases the results were the same when exonuclease VII (16,17) or S1 nuclease was used to degrade the single-stranded nucleic acids away from hybridized species.

Cleavage sites at the 3' end of pre-16S rRNA: processing in cell extracts and termini present in vivo.

The probe was labeled at the terminus that lies in the precursor-specific region (Fig. 3, below). This permits one to detect products cleaved away from the mature rRNA sequence.

Fragments present in rRNA preparations from ribosomes of wild-type and RNase III deficient strains are shown in Fig. 3 (left). In strain D10, only a very small fraction of the RNA was present in precursor form (P; lane 6). Isolated ribosomes apparently contained most of the 3' RNA termini in mature form. However, long pre-RNA (P) and some fragments produced after RNase III cleavage (R) were detected in the total cellular RNA (Fig. 3 right, lanes 3 and 4). Similar to results at the 5'end, some heterogeneous and much smaller metabolic products of the piece of RNA cleaved away by RNase III were also present (Fig. 3 right, lanes 3 and 4).

In contrast, rRNA preparations from ribosomes of the RNase III-deficient strain contained a prominent amount of long precursors (P) and a heterogeneous population of at least four fragments generated in varying amounts (Fig. 3 left, lane 2). As expected, no species arising from cleavage at the RNase III site was present in RNA from the ABL1 strain (lane 2). From the sizes and comparisons with known rDNA sequence, one can infer that fragments may be produced by cleavage at the accepted mature (M) 3' terminus at nucleotide 3059 in the rrnB sequence (14). Other metabolic products of fragment M were 5 (C), 10 (B), and 14-23 (A) nucleotides out from the mature terminus. Only small amounts of the intact species (M) cleaved directly from the precursor, or of fragments B and C, were seen. Due to their rapid metabolism into smaller fragments (bracketed area in Fig. 3 right), fragment M and other fragments B and C were difficult to detect in total cellular RNA (Fig. 3 right, lanes 1 and 2).

The treatment of ribosomes with the soluble protein fraction (1M ribosome wash) from the RNase III-positive strain produced a species corresponding to the RNase III cleaved fragment (R). One

can infer that the fragment most probably arises from RNase III action (18). As treatment was prolonged (Fig. 3 left, lanes 4 and 5, and data not shown), fragments of 34 to 64 nucleotides and an increasing number of very small bits were formed. These fragments are comparable to those seen in preparations of RNA from whole cells (cf. Fig. 3 right, lanes 3 and 4).

When a ribosome wash from the RNase III-deficient strain was used instead of that from the wild-type strain, no fragment was produced with the mobility of the product of RNase III action; but the large fragment (M) released when the mature terminus was formed (lane 3) was again progressively degraded to the same smaller bits seen in preparations of RNA from whole cells (cf. Fig. 3 right, lanes 1 and 2).

The heterogeneous termini at the 3'end probably resulted from enzyme action *in vivo* rather than from the "nibbling" of a single terminus during S1 nuclease assays *in vitro*, for two reasons. First, the same termini in similar proportions were seen after reaction with S1 over a range of concentrations and temperature. Second, the reaction was also carried out with exonuclease VII (16,17) instead of S1 nuclease, with similar results (data not shown).

DISCUSSION

Figure 4 indicates all of the cleavage sites inferred from the S1 nuclease protection studies. The 16S pre-rRNA is represented in its putative double-stranded configuration (19). Cleavage sites are based on termini detected or fragments released during processing. Endonucleolytic cleavage at the mature terminus (M, bold arrow) and other cleavages at A,B, and C (arrows) at both the 5' and 3' ends are indicated, as are RNase III cleavage sites (R).

The major inference is that a single cleavage can generate the mature terminus from a very long precursor, as discussed below. Dotted arrows (Fig. 4) indicate probable cleavages that then occur during metabolic degradation of the released fragment (M) or during early processing in the RNase III-deficient strain.

Differences in the processing of pre-16S and pre-23S rRNA.

1) Initial RNase III cleavages are indispensible for 23S

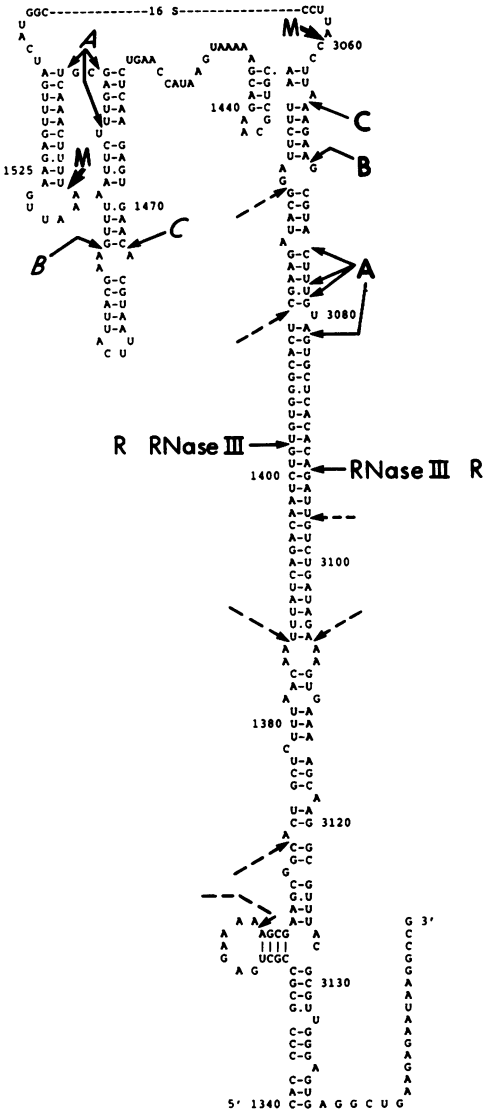

Figure 4. Location of observed cleavage sites during metabolism of pre-16S rRNA (see text). The 16S pre-rRNA is represented in its putative double-stranded configuration (19). Cleavage sites are based on termini detected or fragments released during processing (see text). P denotes precursor molecules detected by full protection of the probe. Dotted arrows indicate probable cleavages during metabolic degradation of the released fragment (M) or during early processing in the RNase III-deficient strain.

rRNA formation, and in their absence processing fails (2,7). We infer that the processing of 23S pre-rRNA is ordered, with obligate cleavage by RNase III before any maturation can occur (3). In contrast, 16S pre-rRNA matures at the same rate whether or not RNase III cleavages have occurred (2, and data here), and thus shows no order of reactions like that for 23S rRNA.

2) Correspondingly, in 23S pre-rRNA, processing, assessed by detecting the termini of precursor-specific RNA sequences, seems to arrive at the mature termini in steps, releasing fragments by a succession of endonucleolytic cleavages at the 5′ end and a combination of endo- and exonucleolytic action at the 3′ end (3). In contrast, in both wild-type and RNase III-deficient strains, the extra precursor sequences at both 5′ and 3′ ends of 16S pre-rRNA seem to be removed by direct endonucleolytic cleavage at or very near the mature terminus, with the release of precursor-specific fragments (Fig. 4). In the studies reported here, this is clearest for the 5′ terminus in vitro and in vivo in Figs. 1 and 2; for the 3′ terminus, it is only clear in vitro, as in Fig. 3 left.

The simplest interpretation, though it is not proven, is that one pathway to mature 16S rRNA is by such direct cleavage of long pre-rRNA. This analysis is limited by the intrinsic instability of the released fragments, both in cells and in extracts. As a result, rates of cleavage cannot be easily quantitated, and the intact fragment cleaved from the 5′end of 16S pre-rRNA in ribosomes is hard to detect. The intact fragment can be seen more easily in transcripts from DNA constructs with a modified rrnB operon (manuscript in preparation). The results obtained here are, however, sufficient to support directly the suggestions in earlier studies that the 5′ precursor portion of 16S pre-rRNA can be removed by an apparently endonucleolytic action (20); and that the 3′ portion may also be removed as an intact fragment (4).

3) 23S pre-rRNA is active enough in ribosomes to support the growth of cells in which all 23S rRNA is immature (7,8). In contrast, ribosomes containing pre-16S rRNA have been found to be inactive in translation (21,22), so that processing very likely must precede function.

Maturation can be carried out in cell extracts. For both 16S (Figs.1 and 3 right) and 23S rRNA (3,9), mature termini can be formed with either ribosomes or polysomes as substrate. In all cases, the released fragments were subsequently degraded by other enzymes, probably by a mixture of exonuclease and endonuclease action. For 16S pre-rRNA, this process yielded products like fragments A, B, and C (Figs. 1 and 2 right) from the 5′ end and A-C (Fig. 3) from the 3′ end. Treatment of ribosomes with different fractions of the cell extract support this notion: the M fragment and other large precursor-specific species appeared and then disappeared on longer treatment (data not shown). It is also evident that in the steady-state, species that were present at very low levels in the wild-type strain were much more prominent in the RNase III-deficient strain -- especially those produced downstream of the 5′ RNase III site and upstream of the 3′ RNase III site (Fig. 4). This may result from the action of enzymes which have a lower affinity or are present at a lower level than RNase III, and are usually preempted by its action in wild-type cells.

Coregulation of processing and ribosome function? 30S ribosomes function as a substrate for processing (5) more than do 50S ribosomes (9); but all rRNA (16S, 23S, and 5S) probably matures after ribosomal particles are formed (8,23,24,25), and precursor sequences may promote a critical conformation in the nascent ribosomes (19).

The direct determination of any requirements of precursor sequence for processing or ribosome formation remains difficult. However, S1 nuclease assays can be used to study the effects on these processes of systematic deletions of 5′ proximal or 3′ distal precursor sequences. They also provide a simple assay for the purification of activities that produce 5′ and 3′ mature termini.

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