Maturation of 23S ribosomal RNA requires the exoribonuclease RNase T

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ABSTRACT

Ribosomal RNAs are generally synthesized as long, primary transcripts that must be extensively processed to generate the mature, functional species. In *Escherichia coli*, it is known that the initial 30S precursor is cleaved during its synthesis by the endonuclease RNase III to generate precursors to the 16S, 23S, and 5S rRNAs. However, despite extensive study, the processes by which these intermediate products are converted to their mature forms are poorly understood. In this article, we describe the maturation of 23S rRNA. Based on Northern analysis of RNA isolated from a variety of mutant strains lacking one or multiple ribonucleases, we show that maturation of the 3' terminus requires the action of RNase T, an enzyme previously implicated in the end turnover of tRNA and in the maturation of small, stable RNAs. Although other exoribonucleases can participate in shortening the 3' end of the initial RNase III cleavage product, RNase T is required for removal of the last few residues. In the absence of RNase T, 23S rRNA products with extra 3' residues accumulate and are incorporated into ribosomes, with only small effects on cell growth. Purified RNase T accurately and efficiently converts these immature ribosomes to their mature forms in vitro, whereas free RNA is processed relatively poorly. In vivo, the processing defect at the 3' end has no effect on 5' maturation, indicating that the latter process proceeds independently. We also find that a portion of the 23S rRNA that accumulates in many RNase T⁻ cells becomes polyadenylated because of the action of poly(A) polymerase I. The requirement for RNase T in 23S rRNA maturation is discussed in relation to a model in which only this enzyme, among the eight exoribonucleases present in *E. coli*, is able to efficiently remove nucleotides close to the double-stranded stem generated by the pairing of the 5' and 3' termini of most stable RNAs.

Keywords: 3' terminus; polyadenylation; ribonuclease; RNA processing

INTRODUCTION

Ribosomal RNA genes are usually organized in large operons encoding multiple rRNA species, and their transcription generates long primary transcripts that must undergo extensive processing to form the mature, functional rRNAs (Srivastava & Schlessinger, 1991). Thus, in *Escherichia coli*, each of its seven rRNA operons encodes 16S, 23S, and 5S rRNA that are cotranscribed as a 30S precursor molecule. In addition, tRNA is present in the spacer region between the 16S and 23S RNAs, and another tRNA is often found downstream of the 5S RNA. It is known that these large precursors are converted in a series of processing steps to the mature RNA species. However, relatively little information is available about the enzymes involved in the process or their mode of action (reviewed by Srivastava & Schlessinger, 1990a; Deutscher, 1993a).

23S rRNA is separated from the other parts of the 30S transcript by the endonuclease RNase III, which cleaves at a double-stranded region flanking the mature RNA (Bram et al., 1980). RNase III action is obligate for the proper maturation of 23S rRNA because in its absence, normal 3' and 5' termini are not formed (King et al., 1984). The RNase III cleavage produces processing intermediates containing seven to nine extra residues at the 3' end of 23S rRNA and either three or seven extra residues at its 5' end (Bram et al., 1980; Sirdeshmukh & Schlessinger, 1985a). The extra residues at the 3' end are thought to be removed exonucleolytically (Sirdeshmukh & Schlessinger, 1985a, 1985b), whereas maturation of the 5' end is probably carried out by an endonuclease and requires conditions of protein synthesis (King et al., 1984; Srivastava & Schlessinger, 1988).

Despite considerable effort devoted to understanding processing of 23S rRNA, the enzymes responsible for generating its mature termini were not known (Deutscher, 1993a; Nicholson, 1997). In this article, we analyze the maturation of 23S rRNA in a variety of

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mutant *E. coli* strains deficient in one or multiple RNases. The results of these studies indicate that RNase T, an exoribonuclease previously shown to be important for the end turnover of tRNA (Deutscher et al., 1985) and for the maturation of tRNA (Reuven & Deutscher, 1993; Li & Deutscher, 1994, 1996), 5S rRNA (Li & Deutscher, 1995), and a number of other small, stable RNAs (Li et al., 1998a), is also required for the formation of the mature 3' end of 23S rRNA. The role of other exoribonucleases in 3' processing of 23S rRNA and the relation of 5' to 3' processing are also described.

RESULTS

Strategies used to study 23S rRNA processing

As mentioned above, RNase III cleavage produces 23S processing intermediates with extra nucleotides at both their 3' and 5' ends. Eight exoribonucleases are now known in *E. coli*, all of which act in the 3' → 5' direction (Deutscher, 1993b). In order to ascertain whether any of these enzymes participate in removal of the extra residues present at the 3' end of the 23S rRNA precursor, mutant strains defective in one or more of the exoribonucleases were examined for possible accumulation of processing intermediates. We took advantage of a site-directed RNase H cleavage method (Inoue et al., 1987; Lapham & Crothers, 1996; Yu et al., 1997) to cut 23S rRNA at a position close to its 3' end to

identify products at single-nucleotide resolution. For this purpose, the chimeric oligonucleotide C23S, containing a run of four consecutive deoxynucleotides and all the rest 2'-O-methyl ribonucleotides, was annealed to a complementary region in 23S rRNA. This results in RNase H cleaving the DNA/RNA double helix at a single position located 5' to the first deoxynucleotide, producing a fragment that extends only 50 nt to the mature 3' end (Lapham et al., 1997, and data not shown). The products derived from the 3' end of 23S rRNA and its processing intermediates were then separated by polyacrylamide gel electrophoresis and detected by Northern blotting (a portion of the gel is shown in Fig. 1). Based on such an analysis, we found that the RNase H cleavage was complete under the conditions used, and thus the short fragments produced accurately represent the distribution of 3' ends of 23S RNA in the total RNA sample. Primer extension analysis was used to examine the 5' ends of 23S RNA.

3' precursors of 23S rRNA accumulate in RNase T-deficient cells

Total cellular RNA was prepared from late log phase cultures of each strain and the status of the 3' end of 23S rRNA in each sample was determined by the procedure outlined above (Fig. 1). No product was seen in the absence of the chimeric oligonucleotide (Fig. 1, lane 1) or RNase H (Fig. 1, lane 2), indicating that the

FIGURE 1. Products from the 3' end of 23S rRNA present in wild-type and various exoribonuclease-deficient cells. Four micrograms of total RNA from various strains were treated with RNase H in the presence of 20 ng of the chimeric oligonucleotide C23S. Samples were then subjected to Northern analysis as described in Materials and Methods. The RNase deficiency of each strain is noted at the top of the gel. The band corresponding to the mature 3' end is indicated by M, and a processing product with eight extra 3' residues is marked by +8.

bands present in the other lanes are specific products generated by the site-directed cleavages of RNase H. One predominant product was observed in wild-type cells (Fig. 1, lanes 3, 8, and 14). This product had the expected size of the mature 3' end of 23S rRNA as determined both by an adjacent DNA sequencing ladder (data not shown), and by sequencing of the reverse transcriptase-coupled PCR products (see below). Minor products one residue longer and one residue shorter than mature were also seen. These products probably represent true longer and shorter 3' ends present in vivo, rather than RNase H cleavages at adjacent sites, because RNase T treatment in vitro shortened the longer ones to the mature size, but did not affect the shorter ones (data not shown). Longer products with up to eight extra residues also were observed at low levels; they presumably represent the steady-state amounts of these processing intermediates in a wild-type cell.

Interestingly, in a mutant cell lacking RNase T (Fig. 1, lane 5), very little mature product is made, and longer products accumulate in large amounts. The two most abundant products are 1 and 3 nt longer than the mature size. Products 2 and 4–8 nt longer and 1 nt shorter are also present, but in much smaller amounts. Cells lacking RNase T in combination with deficiencies in other RNases accumulate similar products (Fig. 1, lanes 6, 10–13, and 17). In contrast, mutant strains lacking only RNase PH (Fig. 1, lane 4) or lacking RNases D, II, and BN in combination (Fig. 1, lane 7) process the 3' end normally. RNase R degrades rRNA substrates well in vitro (Kasai et al., 1977; Cheng et al., 1998). However, its absence has no effect on the maturation of the 3' end of 23S rRNA in vivo, either alone or in combination with RNase PH or RNase T mutations (Fig. 1, lanes 15–17). In cells deficient in RNases PH, D, II, and BN in combination, but retaining RNase T, maturation of the 3' end of 23S rRNA is close to normal (Fig. 1, lane 9). These data strongly suggest that RNase T plays a major role in generating the mature 3' end of 23S rRNA in vivo.

More detailed examination of the data in Figure 1 indicates that although RNase T is required for normal 3' maturation of 23S RNA, other exoribonucleases do contribute to the shortening of the 3' end to some degree. It is evident that even in the absence of RNase T, a small amount of mature 23S RNA can be made (Fig. 1, lanes 5, 6, 10–13, 17). However, the amount of the mature product varies depending on which other RNases are deficient. Thus, the absence of both RNase T and RNase PH leads to less mature RNA and a greater abundance of longer products than in the absence of RNase T alone (Fig. 1, lane 6 compared with lane 5); in contrast, removal of RNases D, II, and BN, in addition to RNase T (Fig. 1, lane 10), has no greater effect than elimination of RNase T by itself (Fig. 1, lane 5). On the other hand, removal of RNases D, II, and BN does result in less mature RNA when both

RNase T and RNase PH are already absent (Fig. 1, lanes 11–13 compared with lane 6). These data suggest that multiple exoribonucleases can contribute to the maturation of 23S rRNA, especially to the shortening of longer intermediates, even though RNase T is by far the most important enzyme in the process. In a separate experiment, it was found that the absence of RNases II, D, BN, and polynucleotide phosphorylase (PNPase), in addition to RNase T, results in no greater defect than obtained by removing only RNase T (data not shown).

3' precursors of 23S rRNA are present in ribosomes

It is generally believed that 23S rRNA is processed in preribosomal particles (Srivastava & Schlessinger, 1990a). Our finding that 23S rRNA in RNase T[−] cells exists largely as precursor molecules with one or three extra 3' residues raises the question of whether these products are also present in the ribosome. To examine this point, ribosomes were prepared from both wild-type and RNase T[−] cells, and rRNAs were isolated. Samples were subjected to site-directed RNase H cleavage and Northern analysis, as above. As shown in Figure 2, 23S rRNA molecules with extra 3' residues predominate in ribosomes from RNase T[−] cells (Fig. 2, lane 4). No difference was found in the distribution of RNA species between total cellular RNA and RNA isolated from ribosomes (Fig. 2, lanes 2 and 4). In wild-type cells, 23S rRNA with a mature 3' end is the major component in both the total RNA and ribosome preparations (Fig. 2, lanes 1 and 3). These data indicate that the majority of ribosomes in RNase T[−] mutant cells carry precursors of 23S rRNA. Inasmuch as RNase

FIGURE 2. Analysis of the 3' fragment of 23S rRNA from total cellular RNA and from ribosomes. The experiment was performed as described in Figure 1.

T⁻ cells grow only slightly more slowly than wild-type (Padmanabha & Deutscher, 1991), ribosomes containing these precursor RNAs must be functional.

Extra 3' nucleotides on 23S rRNA can be removed by purified RNase T

To confirm that RNase T actually is able to catalyze maturation of 23S rRNA, we examined whether purified enzyme could remove the extra 3' residues *in vitro*. Intact ribosomes or free rRNAs from wild-type and RNase T⁻ cells were each incubated with homogeneous RNase T for various periods of time (Fig. 3). As shown in Figure 3A, 23S precursors in ribosomes from RNase T⁻ cells (lane 4) were completely converted to the mature size in just 1 min (lane 5). Longer incubation times (Fig. 3, lanes 6 and 7) or more RNase T (Fig. 3, lane 10) had little additional effect. In the absence of RNase T, there is no change in the length of the precursors (Fig. 3, lane 8), indicating that the processing activity is due solely to RNase T. For both the wild-type and the RNase T⁻ samples, incubation in the presence of excess RNase T leads to a small increase in the amount of product 1 nt shorter than mature.

FIGURE 3. Treatment of ribosomes and isolated rRNA from wild-type and RNase T⁻ cells with purified RNase T. Twenty micrograms of rRNA or the equivalent amount of RNA as ribosomes were mixed with 0.1 μ g of homogeneous RNase T in 20 μ L, as described in Materials and Methods. Reaction mixtures were incubated at 37°C for the indicated time. Eight times as much enzyme was used in the reactions in lanes 9 and 10. **A:** ribosomes; **B:** isolated rRNA. M is the position of the 3' fragment from mature 23S rRNA.

A different situation was observed when free rRNA from RNase T⁻ cells (Fig. 3B, lane 4) was used as substrate. Although the precursors of 23S could be converted to the mature size (Fig. 3B, lanes 5–7), the process was much slower than when the ribosome was used as the substrate. Extra residues were removed gradually, and some precursor remained even after incubation for 10 min. Shorter precursors seemed to be trimmed more rapidly than longer ones. When additional enzyme was used, complete conversion could be achieved (Fig. 3B, lane 10). However, a much larger amount of the shorter (–1 nt) product was also generated. This extra trimming was observed with wild-type RNA as well (Fig. 3B, lane 9). Therefore, processing by RNase T is much more efficient and more accurate with the ribosomal substrate than with isolated RNA.

The 5' end of 23S rRNA is matured independently of the 3' end

The fact that the 3' and 5' ends of 23S rRNA are paired (Bram et al., 1980) presumably would bring the maturation processes of the two termini into close proximity. It was of interest, therefore, to ascertain whether the processing defect at the 3' end would affect maturation of the 5' end. Accordingly, the 5' ends of 23S rRNA from several strains containing or lacking RNase T were examined by primer extension. Figure 4 shows the autoradiogram of the products. The RNAs were from three RNase T⁺ strains that were unaffected in maturation of 23S rRNA (Fig. 4, lanes 1, 2, and 6) and three RNase T⁻ strains that accumulate 3' precursors (Fig. 4, lanes 3–5). As can be seen, all the strains had similar extension products, and in each case, the mature 5' end was the major product. Minor products, 3 or 7 nt longer, were observed in all samples, as was reported previously (Sirdeshmukh & Schlessinger, 1985a). In addition, a product 1 nt longer was also observed in each strain. Inasmuch as the 5' termini of 23S rRNA from RNase T⁺ and T⁻ cells are essentially the same, we conclude that the presence of extra residues at the 3' end has no effect on maturation of the 5' end.

23S rRNA precursors are polyadenylated at their 3' ends

Overexposure of a portion of the Northern blot presented in Figure 1 revealed 3' products in some mutant cells that were up to 10 nt longer than expected from an RNase III cleavage seven to nine residues downstream of the mature 3' end (Fig. 5A, lanes 3, 4, 6–9). Previously, we analyzed sequences at the 3' ends of 23S rRNA using linker-mediated RT-PCR (reverse transcription-coupled polymerase chain reaction; Li et al., 1998b). These data showed that while all the RT-PCR clones from wild-type cells had the expected mature 3' end of 23S rRNA, clones from the examined

FIGURE 4. Analysis of the 5' termini of 23S rRNA in RNase T⁺ and T⁻ cells. Two micrograms of total RNA were annealed to 4 pmol of a ³²P-labeled primer, and the extension reaction carried out as described in Materials and Methods. A DNA sequencing ladder was run alongside the gel to serve as a size marker (not shown). The mature 5' end and longer products are marked by M and the numbers of additional residues, respectively.

mutant strain (RNase T⁻PH⁻D⁻BN⁻) contained extended 3' termini with either precursor sequences up to 8 nt long or precursor sequences followed by an oligo(A) tail. The Northern analysis in Figure 5A indicates that this observation extends to a number of other RNase mutants as well.

The degree of polyadenylation of 23S rRNA precursors appears to vary among the strains examined (Fig. 5A). Products longer than +9 nt are clearly seen in several of the quadruple mutants (Fig. 5A, lanes 7–9). In contrast, one strain lacking RNases T, PH, D, and II showed no polyadenylation (Fig. 5A, lane 10), and *in vitro* assays indicated that this strain is devoid of poly(A) polymerase activity (data not shown). Further work with this strain will be necessary to explain why poly(A) polymerase activity is absent. Nevertheless, these data provide evidence that poly(A) polymerase is necessary for the extended length of the 23S RNA precursors, as was shown previously for mRNA and other stable RNAs (Sarkar, 1997; Li et al., 1998b). Additional evidence is presented in the Northern blot shown in Figure 5B. Elimination of poly(A) polymerase I from a mutant strain lacking RNases T, PH, D, and BN shifted its 23S products to a smaller size, and no RNA longer than expected from the RNase III cleavage could be

seen (Fig. 5B, lane 3 compared with lane 2). Introduction of a plasmid harboring the wild-type poly(A) polymerase I gene into the mutant strain restored formation of the longer products (Fig. 5B, lane 4). These results, taken together with our earlier RT-PCR analysis, indicate that poly(A) polymerase I is responsible for the polyadenylation of 23S precursors.

DISCUSSION

Processing pathway of 23S rRNA

In *E. coli*, maturation of the 30S rRNA precursor begins with RNase III cleavages at potential double-stranded regions flanking the mature 16S and 23S rRNAs (Young & Steitz, 1978; Bram et al., 1980). These "primary processing" reactions produce maturation intermediates of the rRNAs that contain extra residues at both their 5' and 3' termini. Other enzymes are needed to remove these extra 5' and 3' sequences. In this article, we show that RNase T is the primary enzyme responsible for maturation of the 3' end of 23S rRNA. In contrast, RNase T is not involved in the maturation of 16S rRNA (Z. Li, S. Pandit, & M.P. Deutscher, unpubl. observation). In the absence of RNase T, 3' precursors of 23S rRNA accumulate *in vivo*, and purified RNase T can accurately and efficiently complete the maturation process *in vitro*. Because the precursors that accumulate in RNase T⁻ cells are generally shorter than the initial RNase III cleavage products, other exoribonucleases must contribute to the 3' shortening of 23S rRNA precursors to the lengths observed in RNase T⁻ cells. Thus, multiple RNases can each trim the products with seven to nine extra 3' residues, whereas only RNase T can take off the last few nucleotides efficiently. A model accommodating such a two-step exonucleolytic mechanism is presented in Figure 6.

The data presented also show that maturation of the 5' end of 23S rRNA occurs independently of that at the 3' terminus. Earlier work by others had demonstrated that protein synthesis conditions are required for 5' maturation, and that it is probably performed by an endoribonuclease (Sirdeshmukh & Schlessinger, 1985a; Srivastava & Schlessinger, 1988). We have observed a product with one additional nucleotide at the 5' end that was not reported previously. However, because so little is known about 5' maturation, the origin of this product is not understood. RNase III was shown previously not to carry out 5' maturation *in vitro* (Sirdeshmukh & Schlessinger, 1985a). Using primer extension to examine maturation of the 5' end of 23S rRNA, we have found that removal of RNase E or CafA protein (a RNase E homolog) (Wachi et al., 1997), did not alter the extension products when compared to wild-type (data not shown). Thus, we suggest that an activity(ies) other than RNases III, E, or CafA protein is responsible for maturation of the 5' end of 23S rRNA.

FIGURE 5. Longer 3' products of 23S rRNA. **A:** Overexposure of part of the Northern blot in Figure 1 showing that products with more than nine extra residues at the 3' end are present in some mutant strains. **B** shows these products in wild-type (lane 1), in an RNase T⁻PH⁻D⁻BN⁻ mutant strain containing poly(A) polymerase I (lane 2), lacking poly(A) polymerase I (lane 3), and carrying a plasmid restoring poly(A) polymerase I activity (lane 4).

Why RNase T?

RNase T is an important 3' processing enzyme for many stable RNAs in *E. coli* (Li & Deutscher, 1995, 1996; Li et al., 1998a), and it also is responsible for the end turnover of tRNA (Deutscher et al., 1985). All stable RNA species, except 16S rRNA, have base paired 3' and 5' termini, followed by a few unpaired residues

FIGURE 6. Diagram describing the maturation of 23S rRNA. The region flanking the mature termini of 23S rRNA is shown. Sequences representing mature termini are shown in bold letters; precursor sequences are in plain letters. Base-paired residues are matched with vertical bars. Endonucleolytic cleavages are indicated by vertical arrows. Exoribonuclease trimming and polyadenylation reactions are shown by horizontal arrows. Dashed lines indicate less efficient activities. Numbers in circles indicate the proposed order of the reactions.

at the mature 3' end. We have proposed a model in which this terminal stem structure acts as a determinant for accurate exonucleolytic trimming to generate the mature 3' end of the stable RNAs (Li et al., 1998a). RNase T seems to be the only enzyme able to efficiently trim residues close to the stem (Li et al., 1998a), and consequently, it is essential for 5S RNA maturation (Li & Deutscher, 1995) and tRNA end turnover (Deutscher et al., 1985), situations in which residues very near the stem are removed. As shown in Figure 6, the mature 3' terminus of 23S rRNA likewise contains only two unpaired residues at its 3' end. The fact that RNase T also is required for its 3' maturation provides further support for the model. Although other exoribonucleases work inefficiently close to a double-stranded region, they can participate in shortening of longer, single-stranded 3' tails, and this occurs with 23S rRNA as well. It was reported previously that when the 5' end of 23S rRNA contains extra residues (i.e., seven versus three residues), 3' processing is inhibited in vitro (Sirdeshmukh & Schlessinger, 1985a). This is consistent with our model since the presence of these additional 5' residues would lead to an extended terminal stem (Fig. 6). This would stop exonucleolytic trimming before the mature 3' end is reached.

Maturation in the ribosome

Previous work and the results presented here support the conclusion that 23S rRNA processing occurs in the ribosome. Thus, precursors of 23S rRNA can be isolated from ribosome particles, as well as from poly-

some fractions (Srivastava & Schlessinger, 1990b). In RNase III-deficient cells, mature termini of 23S rRNA are not formed (King et al., 1984). However, these cells are viable, although they grow slowly, indicating that such precursors of 23S rRNA must be functional (Srivastava & Schlessinger, 1990a). We have now shown that 3' precursors are the predominant products in RNase T⁻ cells, and they are assembled into ribosomes. These 3' precursors, which are shorter than those in RNase III⁻ cells, are also functional because RNase T⁻ cells grow only slightly more slowly than wild-type. We showed previously that RNase T is essential for the maturation of the 3' end of 5S rRNA (Li & Deutscher, 1995). In its absence, 5S is not matured at its 3' end, and a predominant product with two extra residues accumulates. Thus, in RNase T⁻ cells, precursors to both 23S and 5S would be present in the 50S ribosomal particle. Yet, ribosomes containing these precursors still function quite well.

Our data show that purified RNase T can trim the extra 3' residues from 23S rRNA much more efficiently and accurately when it is in a ribosomal particle than when it is present as isolated RNA. Identical results were obtained previously with 5S RNA (Li & Deutscher, 1995). This suggests that the true maturation substrate *in vivo* is the preribosomal particle. Thus, the correct conformation and/or interaction with ribosomal proteins must be important factors for rRNA processing reactions. In this sense, the maturation of rRNA in the preribosomal 50S subunit should be viewed as occurring in a highly organized RNP substrate acted on by a well-coordinated processing machinery.

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli K12 strain CA2441⁻ (*lacZ*, *trp*, *relA*, *spoT*, *ma*) (Reuven & Deutscher, 1993) was considered wild type for these studies. Most of the exoribonuclease-deficient derivatives of CA2441⁻ were described previously (Kelly & Deutscher, 1992; Li & Deutscher, 1995, 1996). Strains CA2441⁻R⁻, I⁻R⁻T⁻, and I⁻R⁻PH⁻ were constructed by introducing a deletion-interruption mutation of the *rnr* gene encoding RNase R (Cheng et al., 1998) into the corresponding parent strains by phage P1-mediated transduction. The mutations in PNPase, RNase D, RNase T, RNase PH, and RNase R are interruption mutations and are devoid of the relevant activity. The mutations in RNase II and RNase BN have not been defined, but they lead to ~98% loss of the relevant activity. All the strains used in this study are stable, though some multiple RNase-deficient cells grow slowly. Previous work (Kelly & Deutscher, 1992) showed that inactivation of one or more RNases does not lead to overexpression of the remaining enzymes. Plasmid pJL89 harboring the wild-type poly(A) polymerase I gene of *E. coli* was kindly provided by Dr. S. Kushner (Liu & Parkinson, 1989).

Materials

[γ -³²P]-ATP was purchased from DuPont-New England Nuclear. Phage T4 polynucleotide kinase and M-MLV reverse transcriptase were the products of Gibco BRL. *E. coli* RNase T was purified as described (Deutscher & Marlor, 1985; Li et al., 1996). Sequagel for DNA sequencing and Northern analysis was from National Diagnostics. The 2'-O-methyl-RNA/DNA chimera, C23S (5'UGdCdGdCdTUACACACCCG GCC3'), in which all ribo residues are methylated, was synthesized at the Keck Oligonucleotide Synthesis Facility at Yale University (New Haven, Connecticut). C23S is complementary to residues 49–67 from the 3' end of 23S rRNA. All other chemicals were reagent grade.

RNA and ribosome preparations

Cells were grown at 37 °C in YT medium to an A₅₅₀ ~ 1. Total cellular RNA was isolated from 3 mL of culture using the RNeasy kit (Qiagen). Ribosome and ribosomal RNA were prepared as described (Li & Deutscher, 1995).

Site-directed cleavage of 23S rRNA by RNase H

This procedure was carried out as previously described (Yu et al., 1997) with slight modifications. The RNA samples were mixed with an excess amount of the chimeric oligonucleotide, C23S, in 5 μ L, and heated for 5 min at 95 °C and 15 min at 50 °C. The mixture was cooled to 37 °C, and 1 unit of RNase H (Pharmacia) and 2 units of rRNasin (RNase inhibitor, Promega) were added in 5 μ L of buffer containing 40 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, 200 mM KCl, 1 mM dithiothreitol, 10% (w/v) sucrose. The reaction mixture was incubated for 1 h at 37 °C, and the reaction terminated by addition of 20 μ L of RNA gel loading buffer (Li & Deutscher, 1995) and 1 μ L of 5 $\times$ TBE buffer. The mixture was heated for 3 min at 95 °C and cooled on ice to denature the RNA, and analyzed by Northern blotting.

Northern blotting analysis

Northern analysis was carried out according to the procedure described previously (Li & Deutscher, 1995) using a probe complementary to residues 25–48 from the 3' end of 23S rRNA (5'GTACCGGTTAGCTCAACGCATCGC3'). Samples were loaded on a 6% polyacrylamide-urea (8.3 M) sequencing gel. Electrophoresis was carried out at 1,700 V until the xylene cyanol dye had migrated 20 cm. The RNA was blotted to a GeneScreen Plus membrane (New England Nuclear), and hybridized with ³²P-labeled probe at 50 °C.

Treatment with purified RNase T

Ribosome and free RNA samples were treated with purified RNase T as described (Li & Deutscher, 1995; Li et al., 1998a). Samples were incubated at 37 °C for the indicated length of time. The reaction was stopped by diluting 3 μ L of the mixture into 100 μ L of stop buffer (50 mM Tris-Cl, pH 7.5, 10 mM MgCl₂, 100 mM NH₄Cl, 6 mM β -mercaptoethanol, 2 mM EDTA,

1% SDS), and extracted once with buffered phenol/chloroform/isoamyl alcohol (25:24:1), and once with chloroform/isoamyl alcohol (24:1). The RNA was then precipitated with 1/10 volume of K-Acetate (3 M, pH 5.2) and 2 volumes of ethanol, washed twice with cold 70% ethanol, and dried. The RNA was dissolved in water and subjected to the oligonucleotide-directed RNase H treatment as described above. RNase T activity was determined as described (Deutscher & Marlor, 1985).

Primer extension analysis

Primer extension was carried out as previously described (Li & Deutscher, 1995). An oligonucleotide (5'CCTTCATCGCCTCTGACTGCCA3') complementary to residues 34–55 at the 5' end region of 23S rRNA was used as the primer. The extension products were separated by electrophoresis on a 6% sequencing gel, and were detected by autoradiography.

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