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RNase G (CafA protein) and RNase E are both required for the 5′ maturation of 16S ribosomal RNA

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In *Escherichia coli*, rRNA operons are transcribed as 30S precursor molecules that must be extensively processed to generate mature 16S, 23S and 5S rRNA. While it is known that RNase III cleaves the primary transcript to separate the individual rRNAs, there is little information about the secondary processing reactions needed to form their mature 3′ and 5′ termini. We have now found that inactivation of the endoribonuclease RNase E slows down *in vivo* maturation of 16S RNA from the 17S RNase III cleavage product. Moreover, in the absence of CafA protein, a homolog of RNase E, formation of 16S RNA also slows down, but in this case a 16.3S intermediate accumulates. When both RNase E and CafA are inactivated, 5′ maturation of 16S rRNA is completely blocked. In contrast, 3′ maturation is essentially unaffected. The 5′ unprocessed precursor that accumulates in the double mutant can be assembled into 30S and 70S ribosomes. Precursors also can be processed *in vitro* by RNase E and CafA. These data indicate that both RNase E and CafA protein are required for a two step, sequential maturation of the 5′ end of 16S rRNA, and that CafA protein is a new ribonuclease. We propose that it be renamed RNase G.

Keywords: CafA protein/ribosomal RNA/RNA processing/RNase E/RNase G

Introduction

Ribosomal RNAs (rRNAs) are usually synthesized as long primary transcripts that must be converted to their mature, functional forms by a series of processing reactions (Srivastava and Schlessinger, 1991). In *Escherichia coli*, each of the seven rRNA operons is transcribed as a 30S precursor molecule that contains 16S, 23S and 5S rRNA. In addition, tRNA is present in the spacer regions between 16S and 23S RNA, and in some operons, downstream of the 5S RNA as well. Although the processes by which these primary transcripts are converted to the mature RNA species have been studied extensively, there is still scant information about the identity of the enzymes that carry out the processing reactions (reviewed by Srivastava and Schlessinger, 1990; Apirion and Miczak, 1993; Deutscher, 1993).

It is known that the 30S primary transcript is cleaved during its synthesis by the endoribonuclease RNase III resulting in the separation of each of the rRNA species. Subsequent secondary processing reactions are then required to generate the mature 5′ and 3′ termini of each RNA. In the case of 16S rRNA, its precursor is 17S in size, containing an extra 115 residues at the 5′ end and 33 at the 3′ end (Young and Steitz, 1978). In earlier work (Dahlberg *et al*., 1978), it was shown that a product, 16.3S in size, containing 66 extra 5′ residues and a mature 3′ terminus, accumulated in a particular mutant strain. An activity was detected in cell extracts that could convert the 16.3S RNA, in 30S or 70S ribosomal particles, to the mature size. Free RNA was not a substrate. A second activity that removes extra residues from the 3′ end of the 16S RNA precursor also was identified (Hayes and Vasseur, 1976). However, neither of these activities was well characterized, and their relationship to currently known *E.coli* RNases has been unclear (Deutscher, 1993; Nicholson, 1997).

In addition to RNase III, two other endoribonucleases play a role in the maturation of RNAs in the 30S rRNA primary transcript. These are RNase P, which generates the mature 5′ end of all tRNAs (Altman *et al*., 1995), and RNase E, which cleaves on either side of the 5S rRNA in the long transcript to generate a processing intermediate with an extra 3 residues at each end (Ghora and Apirion, 1978). The extra residues at the 3′ terminus are then removed by the exoribonuclease RNase T (Li and Deutscher, 1995). Recently, RNase T was shown also to be responsible for maturation of the 3′ terminus of 23S rRNA (Li *et al*., 1999). Whether any of these enzymes contribute to the maturation of 16S RNA was not known.

In this paper, we analyze maturation of the 5′ terminus of 16S RNA. We show that this process is dependent on two enzymes, RNase E and CafA protein, a homolog of RNase E (Wachi *et al*., 1991; McDowall *et al*., 1993). In the absence of either protein, the efficiency of 5′ processing is reduced considerably and only a limited amount of mature 16S RNA is produced. Moreover, in the absence of CafA, a product with 66 extra 5′ residues accumulates. In a double mutant, under conditions in which both RNase E and CafA are inactive, 5′ processing of 16S RNA is completely blocked. These findings identify two enzymes required for maturation of the 5′ terminus of 16S RNA, and they support the earlier suggestion of Wachi *et al*. (1997) of a functional relationship between RNase E and CafA. In addition, these data strongly suggest that the CafA protein is a new ribonuclease. Based on this information, we recommend that CafA be renamed RNase G, and that its gene be designated *rng*.

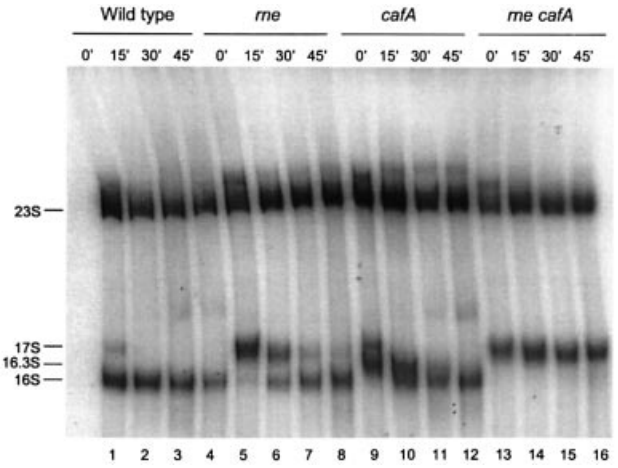

Fig. 1. Analysis of ribosomal RNAs from wild type, *rne*, *cafA* and double mutant strains. Cells were labeled with ^{32}Pi, chased with rifampicin, and RNA isolated and analyzed by SDS–PAGE as described in the Materials and methods. The strains and time after rifampicin addition are shown at the top. The 23S and 16S rRNA bands were identified by co-electrophoresis of rRNA standards (not shown). The 17S and 16.3S precursors to 16S rRNA were tentatively identified based on their migration positions. Detailed characterization of these products is given in subsequent figures.

Results

Maturation of 16S RNA slows in a rne mutant strain

During the course of studies on the physiological role of exoribonucleases in *E.coli*, it became apparent that these enzymes were not required for the maturation of 16S rRNA (unpublished observations). Consequently, we shifted our attention to the possible involvement of known endoribonucleases in the formation of the mature termini of this RNA species. In particular, we were led to consider a role for RNase E. Earlier work from Apirion's laboratory had shown that precursors to 16S RNA accumulated in *rne* mutant strains; however, these workers did not attribute this to a direct effect of RNase E, but to other mutations present in the strains used (reviewed in Apirion and Gegenheimer, 1981; Apirion and Miczak, 1993). Consequently, we decided to re-investigate these earlier observations.

For these studies, the *ams* temperature-sensitive mutant allele of the *rne* gene (Ono and Kuwano, 1980) was transferred to wild-type strain CA244, and 16S RNA synthesis in the wild-type and mutant strains was compared. Cells were initially grown at 31°C, transferred to 44°C to inactivate RNase E in the mutant strain, and then labeled with ^{32}Pi for 20 min. Rifampicin was then added to stop further initiation of transcription. As shown in Figure 1, mature 16S RNA is made very rapidly in wild-type cells. By the end of the labeling period (Figure 1, lane 1), almost all of the 16S-related product is of mature size. A small amount of a longer product, corresponding to the 17S precursor (see below), is also seen, but this disappears after 15 min of chase (Figure 1, lane 2). In contrast, in the *rne* mutant strain, essentially all of the 16S product is present as the precursor form at 0 min (Figure 1, lane 5). This product slowly is converted to the mature size during the chase period, but some precursor is observed even 45 min after the labeling period (Figure 1, lane 8). These data indicate that conversion of the precursor to the mature form of 16S RNA is slowed down dramatically when RNase E activity is absent. However, the block is not complete and mature 16S RNA can be made, indicating either that some other activity also can carry out the maturation reaction or that residual RNase E remains at 44°C.

Maturation of 16S RNA is altered in a cafA mutant strain

Because 16S RNA maturation continued in the *rne* mutant strain, albeit slowly, it was of interest to determine whether another enzyme might also carry out this function. One possibility for such an activity was the CafA protein, which shares extensive sequence similarity with RNase E (McDowall *et al*., 1993) and which had been suggested to have a functional relationship with RNase E (Wachi *et al*., 1997). Accordingly, the *cafA*::*cam*^{r} interruption mutation present in strain GW11 was transferred into strain CA244, and 16S RNA maturation in this strain was examined as above (Figure 1). As with the *rne* mutation, little or no mature 16S RNA was present in the mutant at 0 min (Figure 1, lane 9). Rather, the 17S precursor and a more intense band between 16S and 17S RNA were observed. As will be shown below, this latter band corresponds to the 16.3S RNA previously seen in the 'BUMMER' mutant strain (Dahlberg *et al*., 1978). By 15 min after labeling (Figure 1, lane 10), the 17S RNA was absent, and by 45 min (lane 12) the 16.3S band also had disappeared and the product was mature 16S RNA. These data indicate that the absence of CafA protein also slows down maturation of 16S RNA, and that in this case, a new processing intermediate is observed.

Maturation of 16S RNA is blocked in a rne, cafA double mutant strain

The findings with the *rne* and *cafA* mutant strains suggested that both RNase E and CafA protein participated in the maturation of 16S RNA. To examine this point in more detail, a double mutant strain was constructed, and the maturation of 16S RNA was examined in this genetic background at 44°C. As can be seen in Figure 1, lanes 13–16, under these conditions, maturation of 16S RNA was completely blocked. Careful inspection of Figure 1 reveals that there is a slight shortening of the 17S precursor during the 45 min chase period, which will be explained below, but no 16.3S or mature 16S RNA is made. These data strongly suggest that both RNase E and CafA are required for maturation of 16S RNA.

Synthesis of 16S RNA was also examined at 31°C (data not shown). Under these conditions, in which the temperature-sensitive RNase E remains active but CafA is still absent, the *rne* mutant strain behaves like wild type, and the CafA mutant generates the same products as were made at 44°C. These data support the conclusion that the activities of RNase E and CafA are involved in 16S RNA maturation, and that the effects observed were most likely not a consequence of the use of 44°C or of other mutations in the strains.

Maturation of the 5′ terminus of 16S RNA is affected in mutant strains

To ascertain the specific roles of RNase E and CafA in 16S RNA maturation, we undertook an analysis of the

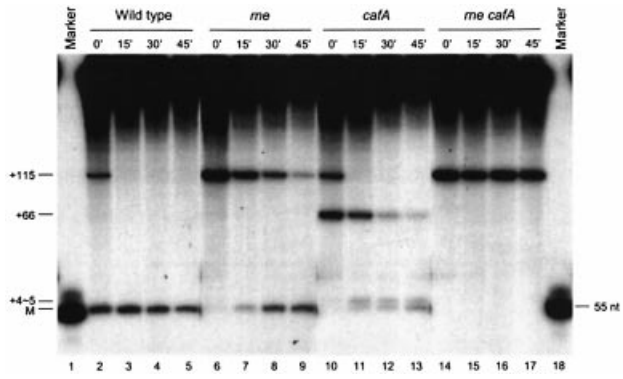

Fig. 2. Analysis of the 5′ termini of 16S RNA and related products. 16S RNA and precursors were isolated from agarose gels and the 5′ termini detected by site-directed RNase H cleavage as described in the Materials and methods. The chimeric oligonucleotide, C16S5 (10 ng), was mixed with the isolated RNA (~10 000 c.p.m.) in 5 µl of solution. Samples were heated at 95°C for 3 min and then at 50°C for 10 min to anneal the oligonucleotide to the RNA. Five microliters of a mixture containing 2 U of RNase H was added and incubation was continued for 60 min at 37°C to obtain complete cleavage of the RNA. After addition of 2 vol. of loading buffer, the products were separated on acrylamide gels as detailed in the Materials and methods. The strains and time after rifampicin addition are shown at the top. Sizes of the 16S 5′ end products are indicated on the left. These were determined from an adjacent DNA sequencing ladder and low molecular weight RNAs (not shown), as well as an *in vitro* transcribed 55 nt RNA marker shown in lanes 1 and 18. M, mature 5′ end.

termini of 16S RNA and of the 16S RNA-related products that accumulated in the mutant strains. The ^{32}P products identified in Figure 1 were isolated and subjected to site-directed RNase H cleavage as described in the Materials and methods. For determination of the 5′ termini, a chimeric DNA/2′-*O*-methyl RNA oligonucleotide complementary to residues 40–57 of 16S RNA was used. Upon annealing to 16S RNA, this chimera directs RNase H cleavage at a single position located 55 residues downstream of the mature 5′ end (Lapham *et al*., 1997; Li *et al*., 1999) and therefore a fragment 55 nt in length would be expected to be produced from any mature 16S RNA present. Precursors to 16S RNA with extra 5′ sequences would be expected to produce longer fragments. Using this technique and an adjacent DNA sequencing ladder or specific RNA markers allowed us to determine the 5′ terminal length to single nucleotide resolution. All the cleavage products observed by this procedure were specific to the 5′ end of 16S RNA and its precursors as no bands were seen in the absence of the chimera or of RNase H (data not shown).

Two products were observed in wild-type cells at 0 min (Figure 2, lane 2). The major product, 55 nt in length, corresponded to the mature 5′ end. The second, longer product was 170 nt in length, or 115 nt longer than mature, and corresponds to the 5′ end of 17S RNA, the immediate product of RNase III cleavage (Young and Steitz, 1978). After 15 min of chase (Figure 2, lane 3), the longer product was no longer present, and only material with a mature 5′ terminus was observed.

The same two products were observed in the *rne* mutant cells (Figure 2). However, in this case, at 0 min only the longer product was present (Figure 2, lane 6). During the course of the chase period, the longer product was gradually converted to the mature size, but even after 45 min (Figure 2, lane 9) some longer product remained. These data show that in the absence of RNase E, maturation of the 5′ terminus of 16S RNA is slowed dramatically. Nevertheless, 5′ maturation still proceeds by what appears to be a single endonucleolytic cleavage at the 5′ terminus, presumably carried out by the remaining activity, CafA (see below).

The pattern of products was quite different in the *cafA* mutant strain (Figure 2). In addition to the 17S product with 115 extra 5′ residues, a major product 121 nt in length, or 66 nt longer than the mature product, was present at 0 min (Figure 2, lane 10). The longest product disappeared by 15 min (Figure 2, lane 11), whereas the product with 66 extra 5′ residues slowly decreased during the course of the 45 min chase. As the longer products disappeared, two other products gradually increased with time. One contained the mature 5′ terminus, and one was a product with 4 or 5 additional 5′ residues. Thus, in the absence of CafA protein, overall synthesis of mature 16S RNA is greatly reduced, a processing intermediate containing 66 extra 5′ residues is formed, and 5′ maturation is less accurate. All of these products apparently are generated by RNase E (see below).

In a double mutant in which neither RNase E nor CafA functions, no 5′ maturation occurs, and only the initial RNase III cleavage product containing 115 extra 5′ residues is observed (Figure 2, lanes 14–17). These results confirm that both RNase E and CafA protein are required for maturation of 16S RNA and that they both contribute to maturation at the 5′ end of the molecule. The data are most consistent with a two step reaction for processing at the 5′ terminus. In the first step, RNase E acts on the RNase III cleavage product to generate an intermediate with 66 extra 5′ residues. CafA then acts on this intermediate to generate the mature 5′ end. This pathway is presented in more detail in the Discussion. However, based on this information, we conclude that CafA protein is a new ribonuclease, and we propose that it be renamed RNase G, and its gene be renamed *rng*.

Maturation of the 3′ terminus of 16S RNA is slightly affected in rne mutant strains

The 3′ termini of 16S RNA and its related products were analyzed by the same method as employed for the 5′ ends. The chimeric oligonucleotide used in this instance was complementary to residues 1437–1455 of 16S RNA, and directed RNase H cleavage 89 nt upstream of the mature 3′ terminus. As shown in Figure 3, two major products were produced in each strain. One product was 89 nt long and corresponds to the mature 3′ end of 16S RNA. The other was 122 nt in length, or 33 nt longer than mature, and corresponds to the product derived from the RNase III cleavage in the 3′ flanking region of 16S RNA (Young and Steitz, 1978). In wild-type cells, the precursor product was present in small amount at the beginning of the chase period (Figure 3, lane 2) and disappeared by 15 min (lane 3). The *cafA* mutant behaved exactly as wild type (Figure 3, lanes 10–13). However, in the *rne* and double mutant strains (Figure 3, lanes 6–9 and 14–17), initially the precursor was the major product and it was converted to the mature size over 15–30 min, explaining the results in Figure 1. These data indicate that while 3′ maturation of 16S RNA can proceed even in the

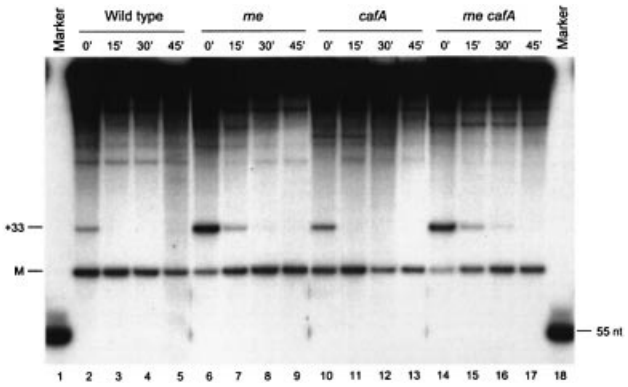

Fig. 3. Analysis of the 3′ termini of 16S RNA and related products. Samples were analyzed as in Figure 2 except that the chimeric oligonucleotide C16S3 was used.

complete absence of 5′ processing (i.e. in the *rne, cafA* double mutant), it is slowed considerably when RNase E is inactivated. The results also suggest that removal of the extra 33 nucleotides at the 3′ terminus occurs by a single endonucleolytic cleavage at the mature 3′ end.

Precursors to 16S RNA are assembled into ribosomes

The accumulation of 5′ unprocessed precursors to 16S RNA in the *rne, cafA* mutant strain raised the question of the status of these RNA species within the cell. To address this point, ^{32}P-labeled RNA products from wild-type and mutant cells were fractionated by ultracentrifugation on sucrose gradients, as described in the Materials and methods, to determine whether or not they were present in ribosomal particles. Samples were prepared 30 min after addition of rifampicin following a 20 min labeling period with ^{32}Pi at 44°C. Gradients were run both at high and low Mg^{2+} to examine 70S ribosomes and 50S and 30S ribosomal subunits. The identities of the various peaks were ascertained from ^{3}H-labeled standards present in the same tubes, and also from their RNA content presented below.

As shown in Figure 4, there is essentially no difference in the ^{32}P ribosome profile between wild-type and double mutant cells either at high or low Mg^{2+}. In both cells, after a 30 min chase period, the bulk of the ^{32}P label in high Mg^{2+} is in 70S ribosomes rather than in subunits. At low Mg^{2+}, both cells have similar amounts of labeled 50S and 30S subunits. These data show that newly synthesized 70S ribosomes can be assembled in the double mutant cells, and since no mature 16S RNA is present under these conditions (Figures 1 and 2), they suggest that the 5′ unprocessed 16S precursor is assembled into 30S subunits and subsequently into 70S ribosomes.

To confirm this conclusion, RNA was isolated from the 70S, 50S and 30S peaks of the high Mg^{2+} gradient and analyzed on 3% polyacrylamide gels (Figure 5). As expected, in wild-type cells the 70S peak contained both 23S and 16S RNA, while only 23S RNA or 16S RNA was present in the 50S or 30S subunits, respectively. In contrast, in the double mutant, 16S RNA was totally absent and instead 17S RNA precursor was present in both the 70S ribosome and the 30S subunit. These data indicate that the 17S RNA species is assembled into ribosomes despite the absence of any 5′ processing. In fact, no free 16S precursor RNA was detected on the sucrose gradient (data not shown).

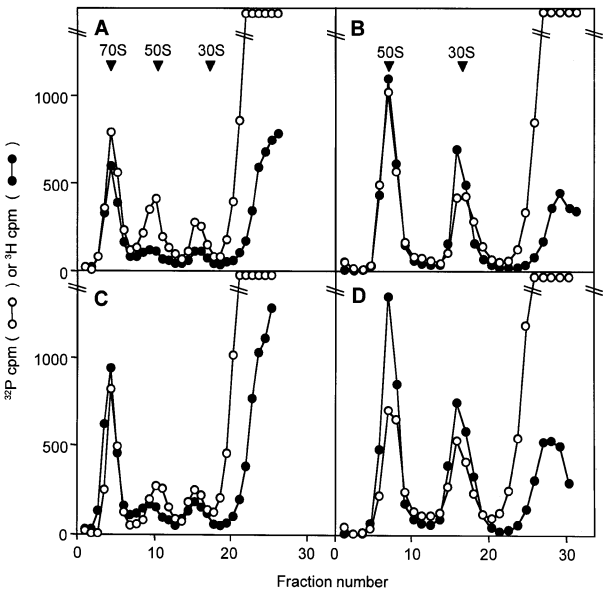

Fig. 4. Sucrose gradient analysis of ribosomes from wild type, and *rne, cafA* double mutant strains. Cells, labeled with ^{32}P as in Figure 1, were collected 30 min after rifampicin addition. For size markers, wild-type cells were grown in 5 μCi/ml of [^{3}H]uridine at 37°C. Two hundred microliters of a mixture of ^{3}H and ^{32}P cell extracts (S30 fractions) containing ~50 000 c.p.m. of ^{3}H and ~100 000 c.p.m. of ^{32}P were loaded on 5–20% sucrose gradients and centrifuged as described in the Materials and methods. The ^{3}H and ^{32}P radioactivity were determined from a portion of each fraction. (**A** and **B**) Wild type in 10 mM and 0.3 mM Mg^{2+}, respectively; (**C** and **D**) *rne, cafA* mutant in 10 mM and 0.3 mM Mg^{2+}, respectively.

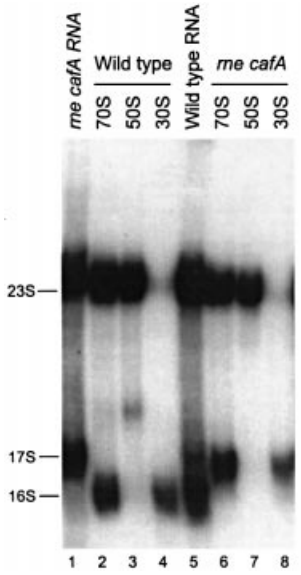

Fig. 5. Analysis of RNA present in the ribosomal particles from wild type, and *rne, cafA* double mutant strains. RNA from the ribosome peak fractions of the sucrose gradients shown in Figure 4A and C was fractionated on 3% acrylamide gels, as in Figure 1. RNA from wild-type ribosomes (lane 5) and from ribosomes of the double mutant (lane 1) served as markers.

16S precursors can be processed in vitro

We examined whether the precursors of 16S rRNA that accumulate in the mutant strains are substrates of CafA

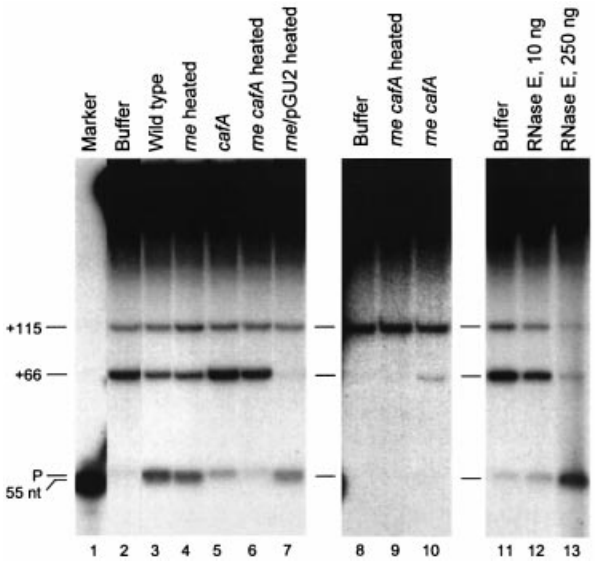

Fig. 6. Processing of 16S rRNA precursors by extracts and RNase E *in vitro.* Approximately 0.5 μg of ^{32}P-labeled ribosome substrates were incubated in 10 μl reaction mixtures with 2 μg (0.4 μg in lane 7) of S100 cell extract for 5 min, or with partially purified RNase E for 15 min using conditions described in the Materials and methods. 16S-related RNAs were isolated, digested with RNase H, and separated on a 6% polyacrylamide gel as in Figure 2. P indicates the position of the 5′ product generated in wild-type extracts. Lanes 1–7 show substrate from the *cafA* strain treated with various cell extracts. The *rne*/pGU2 extract contains CafA protein overexpressed from the plasmid. Lanes 8–10 show substrate from the double mutant strain treated with cell extracts. Lanes 11–13 show substrates from the *cafA* strain treated with partially purified RNase E.

protein and RNase E *in vitro*. Ribosome particles containing labeled precursors, isolated from either the *cafA* strain or the *rne, cafA* strain, were treated with cell extracts or partially purified RNase E. After treatment, the 16S-related RNAs were isolated and their 5′ termini analyzed by the site-directed RNase H cleavage method.

As shown in Figure 6, lane 2, substrate isolated from the *cafA* strain contained predominantly the +115 and +66 nt 5′ precursors, with the latter present in a larger quantity. Treatment of this material with wild-type cell extract led to a reduction in the amount of the +66 nt precursor and the formation of a product approximately the size of the mature 5′ fragment (Figure 6, lane 3). No change in the +115 nt precursor was apparent. Identical results were obtained with a heated extract from the *rne* mutant strain (Figure 6, lane 4), demonstrating that the conversion of the +66 nt precursor does not depend on RNase E. However, elimination of CafA protein in either the *cafA* mutant (Figure 6, lane 5) or the *rne, cafA* double mutant (lane 6) essentially prevented formation of the shorter product, implicating CafA in the process. Moreover, overexpression of CafA protein (Figure 6, lane 7) led to complete conversion of the +66 nt precursor to the shorter form, even with only one-fifth as much extract. Again, the +115 nt precursor was relatively unaffected. These results show that processing of the +66 nt precursor is carried out by CafA, whereas the +115 nt precursor is a poor substrate for the enzyme, in agreement with the *in vivo* data presented above. In addition, these results support the conclusion that the product of the *cafA* gene is, in fact, a new ribonuclease.

Substrate was also prepared from the double mutant strain. As shown in Figure 6, lane 8, this material consists of only the +115 nt product. After treatment with an extract from the *rne, cafA* double mutant strain, a product migrating at the +66 nt position was found (Figure 6, lane 10). This product was absent after heating of the extract to inactivate RNase E (Figure 6, lane 9). This experiment clearly indicates that RNase E converts the +115 nt precursor to the +66 nt product, although this could not be clearly ascertained with the mixed substrate used in Figure 6, lanes 1–7, because of the large amount of the +66 nt product already present before any treatment. However, to substantiate the role of RNase E in 5′ maturation, the mixed substrate from the *cafA* strain was also treated with purified RNase E (Figure 6). Incubation with a low level of RNase E slightly reduced the amount of both the +115 nt and the +66 nt precursors with a concomitant increase in the amount of processed product (Figure 6, lane 12). Treatment with a large amount of enzyme converted the majority of the +115 nt and +66 nt precursors to the processed product (Figure 6, lane 13). These data demonstrate directly that RNase E acts efficiently on the +115 nt precursor, and also on the +66 nt precursor when sufficient activity is present, in complete agreement with the *in vivo* observations.

Discussion

In *E.coli*, processing of 16S RNA is initiated by RNase III cleavages in the double-stranded region flanking the mature sequence (Young and Steitz, 1978). The 17S RNA intermediate generated in this step contains extra sequence at each end that must be removed by other enzymes. In this paper, we show that RNase E and CafA protein (now called RNase G) jointly participate in removal of the extra 115 residues present at the 5′ terminus of 16S RNA after RNase III action. Thus, in the absence of either RNase E or RNase G activity, the rate of 5′ maturation is greatly reduced; moreover, in the absence of RNase G, two new products appear. One has an extra 66 residues at the 5′ end, and the second contains four or five extra 5′ nucleotides. In the absence of both RNase activities, 5′ maturation ceases and 17S RNA accumulates. From these data, it appears that RNase E and RNase G are both necessary to carry out the 5′ maturation process.

It is interesting that 3′ maturation depends, to some degree, on efficient 5′ processing. Although a mature 3′ terminus can still be made when 5′ processing is slowed, or even completely blocked, its rate of formation is affected. Thus, in the *rne* single mutant or in the double mutant, incompletely processed 3′ termini are still evident as long as 30 min after the labeling period, whereas in wild type or in the *cafA* (*rng*) strain, 3′ precursors are converted to the mature form within 15 min. We attribute this effect on 3′ processing to the fact that in the 17S rRNA precursor, 26 of the 33 extra 3′ nucleotides have the potential to base pair with 5′ precursor-specific residues (Srivastava and Schlessinger, 1990), and we suggest that the resulting terminal stem is inhibitory to processing at the 3′ end. We propose that once RNase E cleavage occurs, generating the product with only 66 extra 5′ residues, the terminal stem can no longer form and 3′ maturation would then be accelerated.

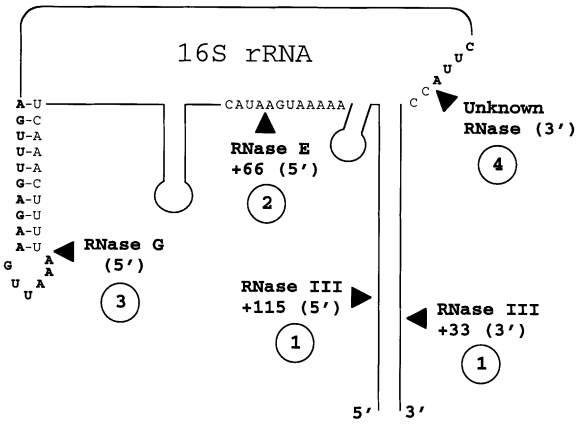

Fig. 7. A model for the maturation pathway of 16S rRNA. Sequences around the mature termini of 16S rRNA are shown; bold letters indicate the sequences in the mature RNA. Ribonuclease cleavages are indicated by arrows. The proposed order of cleavages are marked by numbers in circles. Following RNase III cleavage (step 1), cleavage by RNase E at the 5′ +66 nt position occurs (step 2). Its blockage significantly slows 5′ maturation and also slightly inhibits 3′ maturation. Final maturation at the 5′ end by RNase G (step 3) probably precedes 3′ maturation (step 4) because it was shown that the latter process has a longer half time (Srivastava and Schlessinger, 1990a). Although each step in the process facilitates the subsequent step, it is not obligatory. Thus, while there is a preferred maturation pathway, later steps may occur in the absence of earlier ones, but usually less efficiently (Dahlberg *et al*., 1978; Srivastava and Schlessinger, 1990a; this work).

The identity of the 3′ processing enzyme(s) is not known. Hayes and Vasseur (1976) partially purified an activity that could form the mature 3′ terminus of 16S RNA, but its relation to the known repertoire of *E.coli* RNases is unclear. Inasmuch as no intermediates with lengths between the +33 form and the mature form are observed, even when 3′ processing is slowed down, our data strongly suggest that 3′ maturation is carried out by an endonuclease. However, RNase III, RNase E or RNase G clearly are not involved. Furthermore, 3′ processing is unaffected by the absence of *E.coli* exoribonucleases, alone or in combination (unpublished observations). This contrasts with the 3′ maturation of 23S and 5S rRNAs which are dependent on the exonuclease RNase T (Li and Deutscher, 1995; Li *et al*., 1999). Based on this information, it is probable that 3′ maturation of 16S RNA is accomplished by an as yet undefined enzyme.

From the aforementioned information, we have constructed a probable processing pathway for 16S rRNA (Figure 7). As neither RNase E nor RNase G works efficiently in the absence of the other enzyme, we propose a two step sequential process for 5′ maturation. First, RNase E cleaves in the 5′ region to generate a product with 66 extra 5′ nucleotides. Inasmuch as this product is undetectable in a wild-type cell, its conversion to the mature form must be very fast. We propose that this latter reaction is carried out by RNase G which rapidly cleaves at the mature 5′ terminus of the shortened molecule. In its absence, the product with an extra 66 residues accumulates. Under conditions in which the initial RNase E cleavage does not occur, RNase G will still cut at the mature 5′ terminus, but the reaction is much less efficient leading to the lower rate of 16S RNA maturation observed. Likewise, in the absence of RNase G, RNase E can generate some molecules containing the mature 5′ terminus, but again the reaction is slow, and in this case an incorrectly processed product with four or five extra 5′ residues also is formed. It should be noted that both the RNase E and RNase G cleavages are in AU-rich regions. As mentioned above, maturation of the 3′ terminus takes place following the initial RNase E cleavage in the 5′ sequence and the consequent disruption of the terminal stem region. Additional support for this processing scheme was obtained from *in vitro* studies using extracts or purified RNase E.

This scheme for maturation of 16S RNA envisions an ordered series of processing steps that depends on both the specificity of the enzymes involved and on the RNA structure generated by each reaction. Efficient processing occurs when each enzyme is present at the proper level to ensure that products flow through the pathway in a way that provides the correct substrate for the next reaction. When this delicately balanced system is perturbed, for example by altering the level of one of the enzymes, secondary reactions may come into play, incorrect products may be made, and the overall rate of formation of the correct product may drop precipitously. However, we do not understand why removal of the extra 115 nucleotides at the 5′ terminus of 16S RNA occurs in two steps and requires two related but distinct RNases.

The data presented here also allows us to draw some conclusions about earlier work in this area. For example, in the studies of Dahlberg *et al*. (1978), a 16.3S product with 66 extra 5′ residues was found to accumulate in the BUMMER mutant strain. Treatment of this product *in vitro* with an activity, termed M16, slowly converted it to a mixture of the mature form and one with a few extra 5′ residues. Based on this information, it is probable that the BUMMER strain is defective in RNase G activity and that RNase M16 is related to RNase E. In fact, the reported size of RNase M16, 70 kDa, is the same as that of certain preparations of RNase E (Misra and Apirion, 1979). It was shown earlier that the 17S precursor to 16S RNA accumulates in a *rne* mutant strain, although at that time the effect was attributed to a second mutation affecting protein synthesis, and only secondarily, 16S RNA maturation (Apirion and Gegenheimer, 1981; Apirion and Miczak, 1993). Based on our data, it is more likely that the accumulation of 17S RNA was a direct consequence of the absence of RNase E activity.

Finally, the data presented in this paper suggest the presence of an additional endoribonuclease in *E.coli*, the CafA protein, which we have renamed RNase G. It is not surprising that the CafA protein turned out to be an RNase as it was shown to have significant homology to RNase E (McDowell *et al*. 1993) and a mutation in the *cafA* (*rng*) gene was found to accentuate a *rne* mutation (Wachi *et al*., 1997). From the information provided here, it is clear that RNase E and RNase G have overlapping but distinct specificities. However, the biological role of RNase G may be more limited than that of RNase E because interruption mutations lacking this activity grow essentially normally (Wachi *et al*., 1997; Z.Li, S.Pandit and M.P.Deutscher, unpublished observations), whereas RNase E is essential for cell growth (Apirion and Lassar, 1978; Ono and Kuwano, 1980). On the other hand, RNase G may also participate in processes other than RNA metabolism, as its overexpression results in an effect

on cell division and in the formation of cytoplasmic axial filaments (Okada *et al*., 1994).

The existence of RNase G may also help to explain the persistence of certain RNA maturation or RNA decay processes in *rne* mutant strains. These had been thought to possibly be due to residual RNase E activity at the non-permissive temperatures. RNase G may also bear a relation to the activity formerly known as RNase K (Nilsson *et al*., 1988; Lundberg *et al*., 1990). Initially, it was thought that RNase K was distinct from RNase E, but subsequent studies led to the conclusion that it was due to a proteolytic fragment of the latter enzyme (Lundberg *et al*., 1995). Inasmuch as purified RNase K has a size (55–60 kDa) close to that predicted for RNase G (55 kDa), and both of these activities have specificities overlapping that of RNase E, the possibility arises that RNase K activity was really a manifestation of RNase G, rather than of RNase E. Further work will be needed to resolve this confusing situation.

The studies presented here reveal the complexity of rRNA maturation. Already as many as five RNases have been implicated in the maturation of the 30S transcript (RNases III, E, G, T and P), and it is likely that more will be identified as we still do not know how the mature 5′ termini of 23S and 5S RNA and the 3′ terminus of 16S RNA are made. Maturation of ribosomal RNA occurs in the context of a preribosomal particle; however, it is not clear how maturation of the RNA is coordinated with assembly of the 30S and 50S particles. We can look forward to more surprises in this important area of investigation.

Materials and methods

Bacterial strains

Escherichia coli K12 strain CA244 (*lacZ, trp, relA, spoT*) (Brenner and Beckwith, 1965) was used as wild type for this study. Strain CA244 *rne*, carrying the *ams* temperature-sensitive allele of the *rne* gene encoding RNase E from strain SK5695 (Babizke and Kushner, 1991), was constructed by phage P1-mediated transduction using a nearby Tn10 insertion as the selectable marker. Strain GW11, carrying a chloramphenicol resistance cassette interrupting *cafA*, and plasmid pGU2, carrying the wild-type *cafA* gene in pBR322 (Wachi *et al*., 1997), were gifts from Dr M.Wachi, Tokyo Institute of Technology, Japan. This mutation was introduced by P1 transduction to generate CA244 *cafA* and CA244 *rne, cafA*.

Materials

^{32}P-orthophosphate was purchased from DuPont-New England Nuclear (Boston, MA). *Esherichia coli* recombinant RNase H was obtained from Promega (Madison, WI). DNA/2′-*O*-methyl-RNA chimeras, in which all the ribose residues are methylated, were synthesized at the Keck Oligonucleotide Synthesis Facility at Yale University (New Haven, CT). The chimera C16S5 (5′-CAdTdGdTdGUUAGGCCUGCCG-3′), used to direct cleavage close to the 5′ end of 16S RNA, is complementary to residues 40–57 of mature 16S RNA. Chimera C16S3 (5′-CCdCdGdAdA-GGUUAAGCUACCU-3′), used for cleavage near the 3′ terminus, is complementary to residues 1437–1455 of mature 16S RNA. All other chemicals were reagent grade.

Cell growth and radioactive labeling

Cells were grown in a low phosphate TB medium (Apirion, 1966) at 31°C with shaking. Overnight cultures were diluted into fresh medium and grown to an A_{550} ≈0.5. The temperature was then shifted to 44°C and cells were shaken for 10 min. Carrier-free ^{32}P-orthophosphate was then added to the cultures (2.5 ml) at a final concentration of 40 μCi/ml, and cells were labeled for 20 min. Rifampicin was added to 400 μg/ml, and shaking at 44°C was continued. At various times after the addition of rifampicin, 0.5 ml portions of the cultures were taken and immediately diluted into 1 ml of an ice cold solution containing 80% ethanol and 1% diethyl pyrocarbonate (DEPC). Cells were collected by centrifugation and resuspended in 100 μl of cell lysis buffer containing 10 mM Tris–Cl pH 7.4, 10 mM Na_2EDTA pH 7.4, 1% SDS, 40% glycerol, 0.1% DEPC, and 0.1% Bromphenol Blue (Gegenheimer *et al*., 1977). Samples were boiled for 1 min prior to RNA analysis. Wild-type cells were labeled with ^{3}H-uridine by diluting overnight cultures 1:100 into YT medium (Sambrook *et al*., 1989) containing 5 μCi/ml of the radioactive nucleoside, and incubating at 37°C until the A_{550} reached ~0.5.

Gel analysis of large RNA species

Boiled cell lysates were directly loaded on a 3% polyacrylamide gel containing 0.2% SDS and 3% glycerol, and electrophoresis was performed as described previously (Gegenheimer *et al*., 1977). Gels were fixed with a solution containing 10% acetic acid and 10% methanol, dried and radioactive bands detected by autoradiography.

Preparative isolation of 16S RNA and its precursors

Boiled cell lysates were loaded on a 1.2% agarose gel and electrophoresis carried out in TAE buffer (Sambrook *et al*., 1989) until the Bromophenol Blue marker had migrated to the bottom of the gel (Kevil *et al*., 1997). The gel was then stained with ethidium bromide, and RNA bands were visualized under UV light. Gel slices containing products 16S to less than 23S in size were excised, and the RNA isolated using the RNaid kit (Bio 101, Inc., Vista, CA). The purity of the RNA preparations were determined by analysis on 3% polyacrylamide gels, as described above, using the original cell lysate as the marker.

Site-directed RNase H cleavage

Reactions were carried out as described previously (Li *et al.*, 1999). Briefly, an excess amount of the DNA/2′-*O*-methyl RNA chimeras were annealed to the isolated RNA, and the hybrid was then specifically cleaved with RNase H. It has been shown that RNase H cleaves the RNA at a single location 5′ to the first complementary deoxynucleotide (Lapham *et al.*, 1997; Li *et al*., 1999; data not shown). The cleavage products were separated on 6% polyacrylamide gels containing 8.3 M urea (National Diagnostics, Atlanta, GA), and were detected by autoradiography.

Analysis of ribosomal particles

^{32}P-labeled ribosomal particles were prepared by opening cells with a French press in TMB medium (10 mM Tris–Cl pH 7.8, 30 mM NH_4Cl, 10 mM $MgCl_2$ and 6 mM β-mercaptoethanol). Ribosomes were separated by ultracentrifugation in 5–20% sucrose gradients as described previously (Zhou and Deutscher, 1997). Briefly, samples were loaded on an 11 ml gradient in TMB buffer and centrifuged for 15 h at 18 500 r.p.m. in a SW41 rotor. To generate ribosomal subunits, samples were centrifuged for 15 h at 21 000 r.p.m. in TMA buffer (the same as TMB except containing 0.3 mM $MgCl_2$). After centrifugation, fractions were collected from the bottom of the tube, and portions were measured for radioactivity in a scintillation counter. ^{3}H-labeled ribosomes from wild-type cells were included in the gradients and served as standards. The various ribosome particles were precipitated by ethanol, resuspended in lysis buffer and separated on a 3% acrylamide gel to examine the size of the RNA present. The 5′ termini of the RNAs were determined by site-directed RNase H cleavages as described above.

In vitro treatment of 16S RNA precursors

Cell extracts were prepared as described previously (Li and Deutscher, 1994). Late log-phase cells, grown in YT medium at 31°C, were collected and resuspended in a buffer containing 10 mM Tris–Cl pH 7.5, 500 mM NH_4Cl, 300 mM KCl, 0.5 mM Na_2EDTA, 0.5% (v/v) Triton X-100, 6 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride and 5% (v/v) glycerol. Cells were opened by two passages through a French press. Portions of the 100 000 *g* supernatant fraction (S100) were stored at –80°C until use. To inactivate the temperature sensitive RNase E, extracts were incubated for 15 min at 45°C.

To prepare ^{32}P-labeled ribosome substrates, labeled extracts described above were centrifuged to remove cell debris. Three milliliters of the 30 000 *g* supernatant fraction were then layered on 1 ml of TMB medium containing 20% sucrose and centrifuged for 2 h at 200 000 *g* in a SW41 rotor. The ribosome pellet was resuspended in small portions in TMB containing 50% glycerol and stored at –20°C.

^{32}P-labeled ribosome substrates were treated with cell extracts in a mixture containing 30 mM Tris–Cl pH 7.5, 10 mM $MgCl_2$, 100 mM NH_4Cl, 60 mM KCl, 0.1 mM Na_2EDTA, 6 mM β-mercaptoethanol,

0.1% Triton X-100 and 6% glycerol. The mixture was incubated at 30°C and the reaction was terminated and RNA precipitated by addition of 2.5 vol. of cold ethanol. The pellet was resuspended in lysis buffer, and 16S-related RNAs were isolated from agarose gels as described above. Purified RNA was then analyzed by the RNase H cleavage procedure to examine 5′ termini.

^{32}P-substrates were also treated with partially purified RNase E (a gift from Dr George Mackie, University of British Columbia) in a mixture containing 25 mM HEPES–KOH pH 7.5, 6 mM $MgCl_2$, 100 mM NH_4Cl, 60 mM KCl, 0.1 mM Na_2EDTA, 0.6 mM β-mercaptoethanol, 0.1 mM dithiothreitol, 8% polyethylene glycol, 0.07% Triton X-100 and 10% glycerol. The reaction was incubated at 30°C, and RNA was analyzed as above.

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