Identification of a Novel RNA Molecule in a New RNA Processing Mutant of Escherichia coli Which Contains 5 S rRNA Sequences*

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A temperature-sensitive mutant of Escherichia coli at the nonpermissive temperature fails to produce normal levels of 5 S rRNA. Instead, a number of larger RNA molecules are accumulated. One of these molecules, a 9 S RNA, contains 5 S rRNA sequences. When the strain is shifted from a nonpermissive to a permissive temperature, radioactive label is lost from the 9 S RNA and appears in 5 S rRNA. The identification of this 5 S rRNA-containing molecule indicates the participation of a new processing ribonuclease (RNase E) in the maturation of rRNA in E. coli. The 9 S RNA was not detected in a wild type strain, indicating that the processing step(s) involved in the formation of 5 S rRNA might be performed before the growing rRNA transcript is terminated.

Studies with a strain of Escherichia coli AB301-105, which contains a large number of mutations, one of which affects the enzyme RNase III (1, 2), showed that in this strain a large molecule, 30 S, can be detected which contains sequences from 16 S and 23 S rRNA (3, 4). In vitro studies showed that RNase III can cleave the 30 S molecule to p16 and p23 rRNA (3, 5).

A series of studies with isogenic strains of E. coli, some of which lack the enzyme RNase III (6-8), suggested that the processing of rRNA is a complex set of events involving a number of enzymatic cleavages, two of which are carried out by RNase III. The cleavages which remove the p23 and the p16 from the growing transcript do not occur in RNase III- cells, and the maturation of the rRNA in such cells is carried out by the remaining processing enzymes. Consequently, one observes, besides the customary 23 S and 16 S ribosomal RNAs, a number of transitory RNA species referred to as 30 S, 25 S, 18 S, and 17 S (6-8).

It was suggested that RNase III is not involved in the maturation of 5 S rRNA since in rnc (RNase III-) cells the metabolism of 5 S rRNA was normal (8). Therefore, it was proposed that a cut be introduced between the 23 S and the 5 S rRNA cistrons by a putative enzyme which was named RNase “E” (8). In order to find a mutation which blocks the formation of this cut, temperature-sensitive mutants were isolated from a single Pl-mediated transduction experiment where the donor was an rnc strain and the recipient was an rnc+ strain (10). Strain N3421 is unable to grow at 43°C, while strain N3422 does. At 44°C, strain N3422 grows very slowly, similar to the growth of the recipient rnc+ strain from which it was isolated.

Preparation of RNA for Fingerprinting—For preparation of the precursor molecule, 20 ml cultures of E. coli (N3422) were grown at 30°C in Tris-based medium (8) supplemented with 0.6% peptone and 0.2% glucose. At A₆₀₀ of 0.4, the culture was transferred to 44°C and 40 min later, $^{32}$P was added (250 µCi/ml); after 40 min, labeling was stopped by adding ice. Cells were centrifuged, resuspended in the same medium (without $^{32}$P), and recentrifuged. Cells were opened in a sodium dodecyl sulfate-containing buffer (Ref. 8; bromphenol blue and diethylpyrocarbonate were omitted from the lysis buffer), and RNA was phenol-extracted. The RNA was precipitated overnight at -20°C with 2 volumes of ethanol from 0.2 M sodium acetate, pH 5.5. RNA was collected by centrifugation at 4,000 x g for 1 h at 0°C, and after vacuum desiccation, was dissolved in 10 mM Tris-HCl, pH 7.5, and radioactivity and RNA concentration were determined. Sample buffer (18) was added to the RNA (one-fifth of its volume) and electrophoresis was carried out in 5/8% tandem preparative polyacrylamide slab gels (170 x 140 x 3 mm). Electrophoresis in the polyacrylamide gel (acrylamide: bisacrylamide; 300:80) lasted for 12 h (10 V/cm), using a Tria/glucose buffer (pH 8.3) containing 0.1% sodium dodecyl sulfate (12). After autoradiography of wet gels, bands were excised and rerun, 9 S in a 12% and 5 S in a 15% polyacrylamide gel. The wet gels were autoradiographed and each band was excised and the slices were finely ground with a glass rod and resuspended in 5 to 9 ml of elution buffer (17). Elution of RNA and determination of purity were carried out as described in Ref. 17. $^{32}$P-labeled 5 S rRNA was prepared in a similar way, from cells of strain N3422 which were grown in minimal medium (11). Labeling was carried out with [5,6-$^{32}$H]Uracil (250 µCi/ml), and cells were labeled overnight.

Fingerprinting—This was performed according to the techniques.* This investigation was supported by National Science Foundation Grant PCM-76-81665. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Results

Strains N3421 (rne) and N3422 (rne') were grown at 30°C, shifted to 44°C, and labeled with $^{32}$P, at 30°C and 44°C, and cell lysates were fractionated in a 5/12% tandem polyacrylamide gel (Fig. 1). It can be seen that RNA metabolism is very similar in the rne+ strain at 30°C and 44°C, and in the rne strain at 30°C. However, in the rne strain at 44°C, a large number of molecules accumulate and in the 9 S region a very prominent band appears, while the material accumulating in the 5 S (rRNA) region is clearly reduced. The various regions of the gel shown in Fig. 1 were quantitated (Table I). As can be seen in Table I, the material accumulating in the 5 S region in the rne strain at 44°C is drastically reduced, while the level of 9 S RNA and other molecules, which migrate between 6 S and 9 S, is greatly increased. The RNA which accumulates below the 5 S region (Fig. 1d, lane 2) does not contain 5 S rRNA sequences. (The prominent molecule which accumulates in rne strains at nonpermissive temperature was designated 9 S since its size was assessed to be about 420 nucleotides. This was determined from its position of migration in gels which contained larger markers, such as 10 S and 16 S RNAs, as well as smaller markers, such as 4 S, 4.5 S, 5 S, and 6 S RNAs. The S value was determined from an empirical formula which relates the size of an RNA molecule to its S value. This formula is effective at least for molecules in the size range from tRNA to 28 S rRNA from eukaryotic cells (21)). The patterns observed in the rne+ strain at 30°C and 44°C were rather similar, but $p_5$ seems to accumulate at 44°C rather than $m_5$. This is probably caused by the reduction in protein synthesis (22). Also, in the rne+ strain at 44°C some material accumulates just below the 9 S RNA. This RNA was purified, digested by T$_1$ RNase, and fingerprinted. It did not contain 5 S RNA sequences. (We chose these conditions since the level of 9 S RNA is higher at 44°C than at 30°C. Also, since strain N3422 accumulates mainly $p_5$ under these conditions, it is easier to distinguish between the lower conformer of the $p_5$ RNA and the molecule(s) which accumulates below it in the rne strain at the nonpermissive temperature. That some of the RNA which accumulates in strain N3422 at 44°C in the 5 S region of the gel is $p_5$ rather than $m_5$ was determined by fingerprinting a T$_1$ RNase digest and redigesting the oligonucleotides from the 5' end by pancreatic RNase. These analyses showed that the 5' end oligonucleotides contained mainly pAUUUG rather than pUG which appears in the mature 5 S RNA (23).)

Since the 9 S region contained the most prominent bands of RNA, we analyzed this material more thoroughly. To facilitate the purification of 9 S, total RNA was subjected to electrophoresis in 5/8% long gels (17 cm). In such a gel, 9 S RNA appears in two bands. The T$_1$ RNase fingerprints of the two bands were identical, and both bands contained 5 S rRNA sequences (Fig. 2). (In Figs. 2 and 3, RNA from both these bands was digested and fingerprinted.) These two bands co-migrated in a 5/12% polyacrylamide gel after RNA purified from each of these two bands was treated with formamide/urea buffer prior to electrophoresis (17, 25). Under these conditions, the 5 S rRNA bands also co-migrate. The 5 S rRNA used for fingerprinting in Figs. 2 and 3 contain material from both 5 S bands. As can be seen in Fig. 2, the 5 S T$_1$ oligonucleotides can be found among T$_1$ oligonucleotides prepared from 9 S.

In order to ascertain if 5 S rRNA sequences are contained
A New RNA Molecule Which Contains 5 S rRNA Sequences

FIG. 2. Characterization of 5 S rRNA and 9 S RNA by T1 RNase fingerprinting. Labeled and carrier yeast RNA were mixed (20 ng) and digested with 10 units of RNase T1 (Sankyo, Calbiochem) in 2 μl of 20 mM Tris-HCl, pH 7.8, 1 mM EDTA (neutralized). Left, 5 S [α-32P]rRNA (0.5 × 10⁶ cpm). The numbers given to each spot correspond to those given by Brownlee (16) for the 5 S rRNA T1 oligonucleotides. In the minifingerprinting technique used here, the oligonucleotides migrate differently than in the analysis used by Brownlee (16). To ascertain the identity of the oligonucleotides, they were eluted and redigested with pancreatic RNase and analyzed by high voltage electrophoresis on DEAE-paper (see Table II). The spots that contain the 5' and 3' ends were not observed as they were not included in the part of the cellulose acetate strip which was used in the second dimension. Some spots which can be clearly observed near Spots 5, 12, and 16 were not numbered since they appear in less than 0.5 M yield. These oligonucleotides are from 5 S rRNA and result from the fact that the E. coli cell contains two major and at least one minor 5 S rRNA species (24). Some spots appear also in the 9 S RNA and two of them which appear in greater molar yields were labeled a and i. Right, 9 S [α-32P]-rRNA (10⁴ cpm). Hatched circles correspond to spots common with 5 S rRNA, as judged by their position in the fingerprint, and they were given similar numbers; the open circles designated by letters contain sequences which do not appear in 5 S rRNA (except for a and i). Some of the larger oligonucleotides were further characterized by digestion with pancreatic RNase (see Table II). In other experiments, the 5' and 3' oligonucleotides were included. They were pUG and CAUon, respectively. As expected, the 5' and 3' end oligonucleotides of the 6 S RNA did not appear among the oligonucleotides of the 9 S RNA.

in the 9 S RNA, both these molecules labeled with [32P] were fingerprinted separately or after mixing (Fig. 3) in a ratio of 5 to 1. As can be seen in Fig. 3, (middle panel) the label was substantially increased only in the 9 S spots which correspond to 5 S rRNA spots. Quantitation of the spots led to a similar conclusion. In a similar experiment, 5 S rRNA was labeled with [3H]uracil mixed with [32P]-labeled 9 S RNA (0.8 × 10⁶:0.1 × 10⁶ cpm) digested with T1 RNase and fingerprinted. All the [3H] counts corresponded to [32P]-labeled oligonucleotides, demonstrating rather convincingly that 5 S sequences exist in the 9 S RNA. (In one experiment, all the plate was cut and counted, while in another experiment the plate was stored for about half a year and then was subjected to fluorography.)

Finally, the large oligonucleotides from a T1 RNase digest of [32P]-labeled 5 S and the corresponding oligonucleotides from a 9 S digest were eluted and redigested with pancreatic RNase and analyzed by high voltage electrophoresis on DEAE-paper. The analysis of such an experiment (Table II) shows that all the large 5 S rRNA T1 oligonucleotides exist in 9 S RNA and that the sequences are those expected in 5 S rRNA, proving beyond reasonable doubt that, in an rne strain at the nonpermissive temperature, 5 S rRNA sequences appear in a larger molecule.

Since strain N3422 (rne+) can resume its growth at 30°C when shifted back from 44°C, we wanted to find out if the 9 S RNA can mature to 5 S RNA. The experiment depicted in Fig. 4 suggests that it can. In this experiment, strain N3421 (rne) was grown at 30°C and shifted to 44°C. After 40 min at 44°C, it was labeled with [32P]; for 30 min; unlabeled potassium phosphate (0.2 M, pH 7.5) was added, and the culture was transferred to 30°C where the 9 S molecule disappeared and 5 S rRNA appeared. Since total incorporation of [32P] was almost completely prevented by the addition of a vast excess of unlabeled phosphate (data not shown), the 5 S RNA could be made only from a pre-existing molecule(s) which contain 5 S sequences. Therefore, this experiment suggests that 9 S can be matured in the cell to 5 S rRNA. (Lanes 2 and 3 in Fig. 4 look somewhat different from Lane 2 in Fig. 1, since they contain less counts then in Fig. 1 and the autoradiography was shorter. Under these conditions, some of the less prominent bands are less visible.)

In this last experiment, when the cells were shifted to 30°C,
protein synthesis resumed and therefore, the enzyme which processed 9 S rRNA to 5 S could have been a newly synthesized enzyme, rather than the pre-existing inactivated enzyme. To distinguish between these two possibilities, we performed an experiment similar to that shown in Fig. 4, but added protein synthesis inhibitors just before the culture was transferred to 30°C. We used streptomycin (200 µg/ml), spectino-

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mycin (200 µg/ml), and chloramphenicol (300 µg/ml) in concentrations sufficient to inhibit protein synthesis by more than 95%, and in all cases, an appreciable amount of maturation occurred. Thus, this experiment suggests that the activity in strain N3421 which affects 5 S maturation can be reversibly heat-inactivated. These two experiments suggest that the mutation rme-3071 directly affects the RNase E enzyme.

TABLE II

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<th>Counts per min</th>
<th>Molar yields</th>
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<th>Spot No.</th>
<th>Oligonucleotides</th>
<th>Counts per min</th>
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* The order of the nucleotides was gauged from the known sequence of the 5 S rRNA.
+ The numbers of the oligonucleotides correspond to those shown in Figs. 2 and 3.
larger oligonucleotides from 5 S rRNA and the corresponding ways, including a redigestion by pancreatic RNase of the but instead accumulates larger molecules one of which, 9 S, cannot produce 5 S rRNA at the nonpermissive temperature, indicating that the 5 S rRNA sequences are unlikely to be at the very end of the rRNA transcript. Electron microscopic studies by Wu and Davidson (29) suggest that the 5 S rRNA is about 200 nucleotides from the end of the 23 S rRNA sequences. Since the 9 S is about 420 nucleotides long, and if it contains sequences from the end of p23 to the 3' end of the rRNA transcript (see Fig. 12 in Ref. 8, or Fig. 4 in Ref. 9), this would suggest that there are about 100 nucleotides between the 5 S rRNA sequences and the 3' end of the rRNA transcript.

At the nonpermissive temperature in the rne strain, a relatively large amount of RNA precursor molecules accumulate (see Fig. 1, ranging in size from about 5 S to 11 S). These could be precursors to tRNA molecules. This view is further supported by the fact that in rne rnp (RNase E- RNase P-) strains a larger number of tRNA molecules fail to mature, as compared with each of the parental strains (30). When a comparison is made with Bacillus subtilis, one can see that in B. subtilis, a precursor to 5 S rRNA which is considerably larger (180 nucleotides) than the mature 5 S rRNA (118 nucleotides; Ref. 31). This RNA can be matured to 5 S rRNA by an activity which was designated RNase M5 (32). In E. coli, no such large precursor was found, and the experiments presented here further support the notion that in E. coli, when protein synthesis is blocked, a 5 S rRNA accumulated is which is only three nucleotides larger than the 5 S rRNA (23). In vitro, extracts of rne+ strains can mature the 9 S RNA to a molecule which is three nucleotides longer than 5 S rRNA (26). Therefore, one possibility is that while RNase E cuts the growing rRNA transcript in E. coli very near the 5' end of the mature 5 S sequences, in B. subtilis, the equivalent enzyme introduces this cut farther from the 5' end of the 5 S rRNA. Another possibility is that B. subtilis does not contain such an enzyme and the precursor which accumulates results from a cut which produces the p23 RNA. (For details of processing of rRNA in E. coli, see Fig. 12 in Ref. 8 or Fig. 4 in Ref. 9.)

The studies reported here together with previous studies (3-9) help to understand the complex processing events which take place in the production of tRNA molecules in the normal bacterial cell. They indicate, as suggested previously (8), the existence of an enzyme RNase E which is involved in the maturation of 5 S rRNA and fulfill a prediction made previously (9) that a molecule which accumulates in rne strains at the nonpermissive temperature contains 5 S rRNA sequences.

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A New RNA Molecule Which Contains 5 S rRNA Sequences

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