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RNA PROCESSING: NEW MUTANTS THAT AFFECT ENDONUCLEOLYTIC PROCESSING OF RNA

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SUMMARY: A strain of Escherichia coli carrying the rne-3071 mutation that affects the RNA processing enzyme ribonuclease E, was mutagenized, and double mutants deficient in RNA processing were isolated. The isolation was based on the appearance of a particular RNA precursor molecule upon infection of an rne mutant with a specific bacteriophage T4 deletion strain. From one of the double mutants the rne mutation was removed, and the new single mutant, designated rng, was examined. In this mutant the maturation of host RNA as well as of bacteriophage T4 RNA is affected. The effect of the rng mutation on RNA synthesis is unique and can be distinguished from the effects of the other established mutations in RNA processing. The effects of the rng mutation can be recognized in vivo and in vitro.

Processing of RNA is an important aspect of cell biology that determines the final concentration of functional RNA molecules in a cell (1-3). In the last few years much of the advance in our knowledge of RNA processing in bacteria came from the analysis of specific mutants of Escherichia coli defective in RNA processing (1-5), and from the analysis of RNase M5 from Bacillus subtilis (6). In order to further our understanding of RNA processing in the bacterial cell, it was necessary to isolate new mutants defective in specific RNA cleavages. In this communication we shall describe the isolation of a new type of an RNA processing mutant in E. coli and show that it affects the activity of the RNA processing enzyme RNase P.

MATERIALS AND METHODS: Most techniques used were according to published procedures as indicated in the figure legends. Cell extracts, S30, were prepared as described by Misra and Apirion (7). The protein concentration of cell extracts were determined by the method of Ehresmann et al. (8). The K band RNA was prepared according to a protocol described by Pragai and Apirion (9). RNase P was assayed in extracts according to Gardiner and Pace (10).
Strains. Bacteriophage T4Δ27 was previously described (11,12). Bacterial strains PP113 (rnp$^+$) and N2020 (rnpA49) were also described previously (13,14). Strain N3771 contains the rne-3071 mutation as well as amber mutations in the his and trp genes.

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RESULTS: Isolation of rng Mutants. In order to isolate new RNA processing mutants, we turned to bacteriophage T4. When bacteriophage T4 infects E. coli cells, one of the early events is the transcription from a single T4 promoter of a segment of the chromosome that contains genes for eight tRNAs and two small RNAs of unknown function (1). A deletion mutant, T4Δ27, is missing seven of the ten genes in this cluster. This is an internal deletion where the first two genes that code for $tRNA^{Gln}$ and $tRNA^{Leu}$ as well as the last gene that codes for one of the small RNAs, Species 1 RNA, remain intact (9,11,12,15).

When bacteriophage T4Δ27 infects a wild type E. coli or an rne mutant at a permissive temperature, the RNA products of the three genes in the T4Δ27 tRNA cluster are accumulated, Species 1 RNA, $tRNA^{Leu}$ and $tRNA^{Gln}$ (Figure 1). However, when the rne mutant is infected at the nonpermissive temperature, the three final RNAs appear in relatively low yields; in addition, two precursor molecules also appear (9). The two precursors have been designated 10.1S and p2Sp1 (precursor number 2 of Species 1 RNA). The 10.1S RNA is a precursor for all the three final RNAs, while p2Sp1 contains only Species 1 plus a tail (3' to Species 1) of about 80 nucleotides which is common to it and to 10.1S RNA (9). It was assumed that p2Sp1 is produced from 10.1S RNA by an endonucleolytic cleavage which is not performed by either one of the three characterized processing enzymes of E. coli RNase III, E or P (9), and therefore we wanted to isolate mutants where this cleavage is abolished and only the 10.1S RNA molecule is accumulated.

In order to accomplish this, the rne mutant N3771 was treated with nitrosoguanidine (16) and cells were plated at 30°C onto rich medium plates and replica plated at 43°C to the same medium. We chose colonies that failed to grow at 43°C after the first replication and also failed to grow at a lower temperature only after two successive replications. The rationale for this strategy was the finding that an rne mutant keeps increasing its mass and protein (but not its cell number) to an appreciable extent (about three-fold) when shifted from 37°C, a permissive temperature, to 43°C, a nonpermissive temperature (17). Moreover,

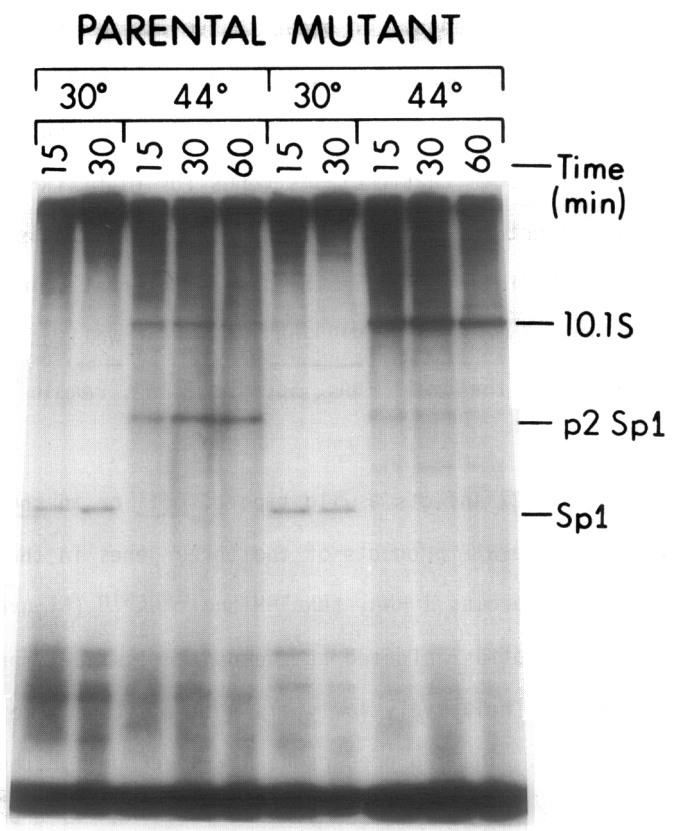

Fig. 1. Formation of RNA in strain N3771 (rne-3071) and a double mutant strain N3915 (rne-3071, rng-3915) after infection with T4Δ27. The cells were grown at 30°C in Tris based medium containing 0.6% peptone (23) and labeled with $^{32}P_i$ (0.2 mCi/ml) at 30°C and 44°C, 5 min after infection. At 44°C cells were infected 30 min after the shift. Samples were withdrawn at the indicated time intervals (time after labeling) and were treated as described by Gegenheimer et al. (23). The picture shown here is of an autoradiogram of the 10% portion of the gel. The top fifth of the gel which consists of 5% polyacrylamide was removed before the gel was dried. The gel contained 7 M urea, and each slot was loaded with about 250,000 counts per minute.

often when two RNA processing mutations are combined, the double mutants are more temperature sensitive than either of the parental strains (18,19).

We screened about 17,000 colonies and concentrated on 554 that were more temperature-sensitive than the parental strain. From these 554 colonies liquid cultures were prepared at 30°C. The cultures were shifted to 43°C, infected with T4Δ27 and labeled with $^{32}P_i$ and the RNA was displayed in a 5%/10% polyacrylamide gel containing 7 M urea. Among the 554 cultures, four mutants were found in which 10.1S RNA was produced while the level of p2Sp1 was either negligible or considerably reduced. We have designated these mutants rne rng

double mutants. RNAs from one of these double rne rng mutants (N3915), in which the level of p2Sp1 was negligible, and the parental rne mutant are displayed in Figure 1. Some of the double mutants grew rather poorly on various media and therefore we crossed them to Hfr strains and isolated recombinants that grew better than the parental strains, but still exhibited the same pattern of RNA accumulation as the parental strains, with or without infection with bacteriophage T4Δ27. One of these strains was designated N3918.

RNA Metabolism in an rng Mutant. In order to remove the rne-3071 mutation from the double mutant strain N3918 and to assess the phenotype of the rng mutation by itself, a P1 phage was propagated on a strain that contains a Tn5 transposon (flaL::Tn5; Ref.20) located near a wild-type rne gene. (We showed, using a single rne-3071 mutant, that the flaL::Tn5 and the rne-3071 mutation are cotransduced by bacteriophage P1.) Strain N3918 (rne, rng) was infected with the P1 lysate and kanamycin resistant colonies were selected. Thirty colonies were isolated and tested for growth at different temperatures. Two of these colonies were more temperature-sensitive than a wild-type strain, but considerably less temperature-sensitive than the parental recipient strain and were assumed to contain the transduced wild-type rne^{+} gene. One of these strains was designated N3919, and further studies were carried out with this strain. RNA metabolism, after infection with T4Δ27, was analyzed in all these thirty strains. While all the 28 strains that remained as temperature-sensitive as the parental recipient strain showed the same RNA pattern as that of the parental rne rng strain (see Figure 1), the two other colonies showed a drastically different pattern of RNA synthesis. This pattern is unique and is different from that observed in a wild-type strain, or in any of the other three known endonucleolytic RNA processing mutants rnc, rne, and rnp. In the rnp mutant no tRNAs are produced, at 43°C, instead a precursor, band K (21) that contains $tRNA^{Leu}$ and $tRNA^{Gln}$ is accumulated. In the rng mutant, at the higher temperature, Species 1 and $tRNA^{Gln}$ fail to accumulate, while $tRNA^{Leu}$ accumulates, in addition, a new molecule similar in size to the K band RNA appears. Structural analysis (M. Gurevitz unpublished observations)

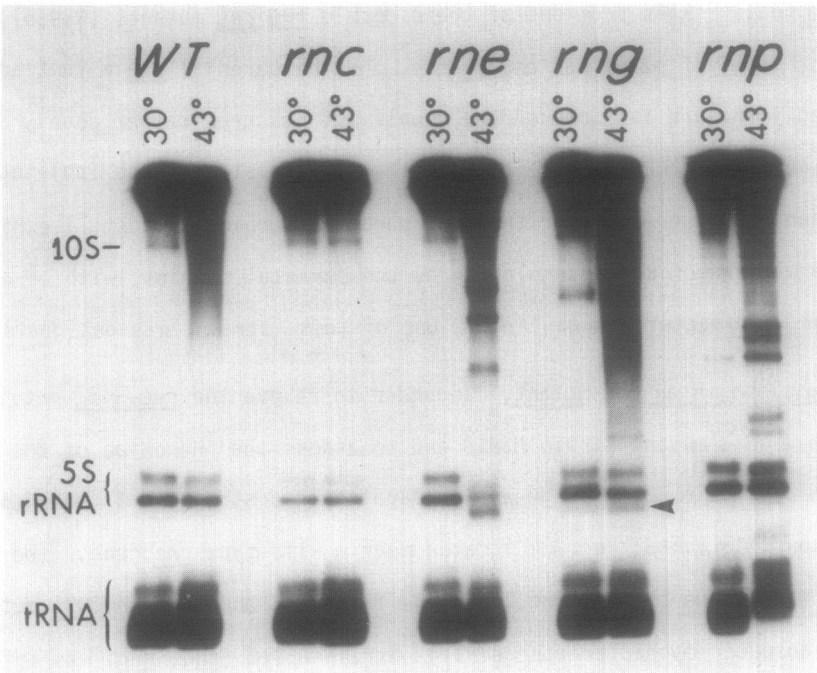

Fig. 2. RNA formation without phage infection. This gel was a 5%/12% tandem polyacrylamide gel and did not contain urea. Labeling, with 0.2 mC/ml, was carried out for 20 min; only the 12% portion of the gel is displayed. For further details see legends to Figures 1. The arrow indicates an RNA precursor that accumulates in the rng mutant at 43°C.

showed that indeed, it is K band RNA. In the double mutant rne rng, only the 10.1S RNA accumulates (see Figure 1).

To find out if the rng-3915 mutation also affects host RNA, we analyzed RNA synthesis in the rng mutant without T4 infection (Fig. 2). Again it was compared to the other three mutants and it can be seen that the rng mutation affects the pattern of RNA synthesis of the E. coli cell in a unique way. The detailed effects of the rnc, rne, and rnp mutations on RNA metabolism in host cells were described in numerous publications (for reviews see: Refs. 1,2,4,5). Here we can see that the rng mutation affects the synthesis of small RNAs. One precursor accumulates below the 5S rRNA (Figure 2) and a number of other putative tRNA precursors accumulate in the region between 5S and 4S. These precursors can be clearly observed by running the RNA in two dimensional polyacrylamide gels.

Activity of Ribonuclease P is affected in an rng mutant. Since the rng mutant accumulates the K band RNA, we decided to find out whether or not it contains

an altered RNase P activity. To test this possibility, extracts were prepared and assayed for RNase P activity before and after heating. As can be seen in Figure 3A, while extracts of a wild type strain contain RNase P activity before and after heating, extracts from an rnp mutant failed to show any activity under any conditions. On the other hand, extracts from the rng strain showed RNase P activity before the heating, while incubation of the extract at 37°C or 43°C abolished the RNase P activity. The inactivation of the RNase P activity is time dependent. (Similar observations were made using two other RNase P substrates that accumulate in an rnp mutant; only these substrates were tested.)

While losing the RNase P activity after preincubation at 43°C might have suggested that the rng mutant contains a thermolabile RNase P activity, inactivation after incubation at 37°C is rather unusual, especially since the rng mutants grow well at 37°C and do not show a mutant phenotype at this temperature with respect to RNA metabolism. Therefore, we tested the effect(s) of a number of inhibitors of proteolytic enzymes on the phenomenon described above. We found that phenylmethylsulfonylfluoride (PMSF) did not inhibit the RNase P activity, but when present in the extracts during the preincubation and the assay, it partially protected the RNase P activity of the rng mutant extract. A similar treatment with PMSF did not restore RNase P activity to rnp extracts (Fig. 3B).

DISCUSSION: The experiments reported here show that using T4 bacteriophage and the rne mutant of the host E. coli, it was possible to isolate new mutants defective in RNA processing. The new mutants were unable to process in vivo the 10.1S RNA after infection with T4Δ27. They seem to have arisen not by defects in a thus far unidentified RNA processing enzyme, but by affecting the activity of a known RNA processing enzyme, ribonuclease P. Since the experiments depicted in Figure 3 show that extracts of the rng mutant are defective in some aspect of ribonuclease P activity, it can be suggested that the rng mutants are similar to rnp mutants. However, close scrutiny of the results point out the differences between an rng mutant and an rnp mutant. While in the latter no mature tRNA or Species 1 are produced at the nonpermissive temperature after infection with T4Δ27, in the former, $tRNA^{Leu}$ is being produced while the

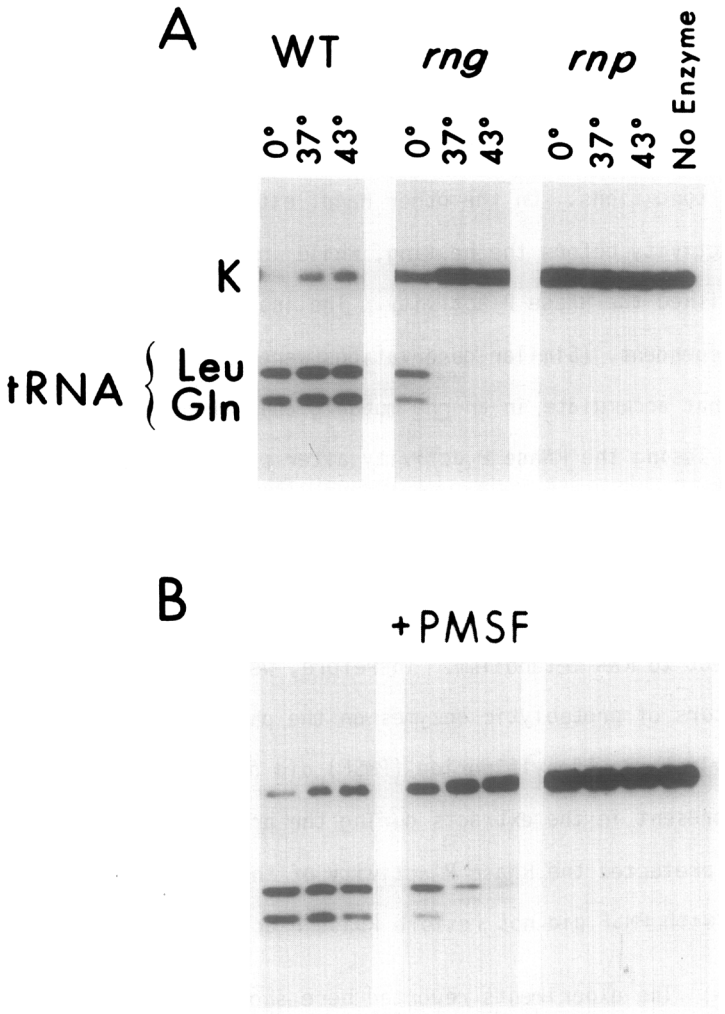

Fig. 3. RNase P activity in extracts of an rng mutant. A. Assays were carried out at 37°C after preincubation of extracts at the indicated temperatures for 30 min. Notice that RNase P activity in the rng mutant can be abolished by preincubation of the extract at 37°C or 43°C. The wild type strain was PP113, the rnp strain was N2020 and the rng strain was N3919. (Strain N3919 was described in the text.) In B the extracts contained 50 mM PMSF (phenylmethylsulfonylfluoride) during the preincubation. Each assay contained 20 µg of S30 protein in a total volume of 40 µl. The products of the reaction were analyzed in a 5%/12% tandem polyacrylamide gel containing 7 M urea. The molecule that appears between $tRNA^{Leu}$ and $tRNA^{Gln}$ is a precursor of $tRNA^{Gln}$. (The K precursor contains two RNase P sites at the 5' ends of each of the two tRNAs. The site in the middle, prior to $tRNA^{Leu}$ is cleaved preferentially by the enzyme resulting in $tRNA^{Leu}$ and a pre-$tRNA^{Gln}$. Only with excess enzyme pre-$tRNA^{Gln}$ is converted to $tRNA^{Gln}$. The pre-$tRNA^{Gln}$ is more susceptible to nucleases in the extract than $tRNA^{Leu}$ and therefore the observed ratios of $tRNA^{Leu}$ to $tRNA^{Gln}$ + pre-$tRNA^{Gln}$ could be higher than one to one.)

accumulation of Species 1 and $tRNA^{Gln}$ is affected (at the nonpermissive temperature). Also the results in Figure 2 show that, as expected, in the rnp

mutant no mature tRNAs are accumulated while in the rng mutant accumulation of tRNA is only somewhat affected. This conclusion was further supported by analyzing the tRNA size RNA molecules in two dimensional polyacrylamide gels.

Moreover, while the rne rng double mutant accumulates 10.1S RNA it does not accumulate any detectable levels of the K RNA precursor at the nonpermissive temperature (Fig. 1); on the other hand the rne rnp double mutant accumulates large quantities of K band RNA at the nonpermissive temperature (see Fig. 1 in Ref. 9). This means that the rne rng double mutant is blocked in an earlier step in the processing pathway than the rne rnp double mutant. Therefore, the possibility that the rng is a leaky rnp mutant is rather unlikely.

The results shown in Figure 3 suggest that RNase P is only indirectly affected in the rng mutant and that the loss of RNase P activity can be at least partially restored by the addition of an inhibitor of proteolytic enzymes (PMSF). These observations can be interpreted by assuming that RNase P exists in the cell and in the cell extract, in a complex which protects it from cellular proteases; that the rng mutation affects one constituent of this complex and thus renders the RNAse P sensitive to a cellular protease(s). The idea that RNAse P as well as other RNA processing enzymes exist in a complex in the cell was already suggested by Jain et al. (22) to explain a number of odd observations.

Finally, if the rng mutation affects indirectly RNase P activity how does it prevent the processing of 10.1S RNA to p2Sp1 in the rne rng double mutants. This could be understood if indeed there is a complex which contains RNase P as well as the enzyme that seperates p2Sp1 from K. (10.1S RNA is composed from K RNA plus p2Sp1, see Ref. 9.) The mutation in the rng gene can affect the activity of the enzyme that cleaves 10.1S RNA to K RNA and to the precursor of Species 1.

Thus, the studies presented here suggest a very close relatedness among some and perhaps all the RNA processing enzymes in E. coli.

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