Escherichia coli RNase M is a multiply altered form of RNase I

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REPORT

Escherichia coli RNase M is a multiply altered form of RNase I

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ABSTRACT

RNase M, an enzyme previously purified to homogeneity from *Escherichia coli*, was suggested to be the RNase responsible for mRNA degradation in this bacterium. Although related to the endoribonuclease, RNase I, its distinct properties led to the conclusion that RNase M was a second, low molecular mass, broad specificity endoribonuclease present in *E. coli*. However, based on sequence analysis, southern hybridization, and enzyme activity, we show that RNase M is, in fact, a multiply altered form of RNase I. In addition to three amino acid substitutions that confer the properties of RNase M on the mutated RNase I, the protein is synthesized from an *rna* gene that contains a UGA nonsense codon at position 5, apparently as a result of a low level of readthrough. We also suggest that RNase M is just one of several previously described endoribonuclease activities that are actually manifestations of RNase I.

Keywords: endoribonuclease; RNA degradation

INTRODUCTION

RNase I is the major nonspecific endoribonuclease in *Escherichia coli* (Shen & Schlessinger, 1982), accounting for >99% of the RNase activity present in crude extracts assayed in the presence of EDTA (Zhu et al., 1990). Although the enzyme is often found in extracts bound to 30S ribosomal subunits (Spahr & Hollingworth, 1961; Datta & Burma, 1972), most of the enzyme actually resides in the periplasmic space in vivo (Neu & Heppel, 1964). RNase I belongs to the T2 superfamily of ribonucleases, whose members are widely distributed throughout nature (Irie, 1997). However, despite extensive work over many years, the physiological role of RNase I remains unclear. In fact, a mutant strain devoid of RNase I remains viable (Zhu et al., 1990).

A number of other nonspecific endoribonucleases, that like RNase I generate 3′ phosphoryl-terminated products in the presence of EDTA, have been reported to be present in *E. coli*. Generally, these enzymes have not been described in sufficient detail to ascertain whether they are distinct enzymes. However, one, termed RNase M, was purified to apparent homogeneity and characterized (Cannistraro & Kennell, 1989; Meador et al., 1990). RNase M was identical in size (27 kDa) to RNase I and displayed a similar, but not identical, tryptic peptide map. In contrast, RNase M differed from RNase I in charge, heat stability, substrate specificity, and subcellular localization. Based on these properties, as well as its purification from an RNase I^- strain, MRE600, it was proposed that RNase M is a second nonspecific endoribonuclease of *E. coli*, though the possibility was left open that it could be a "posttranscriptionally" modified form of RNase I. It was also suggested that RNase M is the endoribonuclease responsible for mRNA degradation in growing cells (Cannistraro & Kennell, 1989).

Despite this work, the existence of a second, nonspecific endoribonuclease was puzzling. First, deletion of the *rna* gene, encoding RNase I, eliminates >99% of the EDTA-dependent activity in extracts (Zhu et al., 1990). Secondly, detailed computer analysis of the *E. coli* genome failed to identify a second gene related to *rna* (Y. Zuo & M.P. Deutscher, unpubl. observations). In view of these discrepancies, and in order to understand how two nonspecific endoribonucleases might participate in RNA metabolism, we have reinvestigated the existence of RNase M. We show here, based on sequence analysis, southern hybridization, and enzyme activity, that RNase M is a mutated form of RNase I, and is not a distinct enzyme. These data also provide

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possible explanations for the reported differences in properties between RNase M and RNase I.

RESULTS

Sequence analysis of the *rna* gene of strain MRE600

RNase M was previously purified from *E. coli* strain MRE600 (Cannistraro & Kennell, 1989), a natural RNase I$^-$ isolate (Wade & Robinson, 1966). To assess RNase I in this strain and to examine whether it might have any relation to the existence of RNase M, we determined the sequence of its *rna* gene that encodes RNase I. As a control, the sequence of the *rna* gene from wild-type strain CA265 was determined in parallel. Each *rna* gene was obtained from multiple, independent PCR reactions that were pooled and gel purified. The results of these sequence analyses are presented in Table 1.

The sequence of the *rna* gene from strain CA265, determined by the methodology used here, was identical to that reported previously (Meador & Kennell, 1990; Zhu & Deutscher, 1992). However, compared to strain CA265, analysis of the *rna* gene of strain MRE600 revealed eight nucleotide changes clustered in three regions of the coding sequence (Table 1). Of these, four are changes in the third position of codons, and leave the encoded amino acid unchanged. Three of the nucleotide changes result in relatively conservative amino acid substitutions, leu to val at position 24, arg to his at position 115, and ala to val at position 266 of the RNase I peptide chain. Surprisingly, the eighth change at nucleotide 15 would result in a UGA termination codon at position 5 of RNase I. Nevertheless, as we will show below, strain MRE600 retains a low level of RNase I activity.

Assuming a small amount of readthrough of the UGA codon in the *rna* gene from MRE600, an RNase I protein would be produced that could explain the existence of RNase M. Thus, Meador et al. (1990) reported that RNase M differed from RNase I in charge and in a few tryptic peptides. The arg-to-his change at position 115 would be expected to result in the loss of one positive charge in the RNase I from MRE600 and earlier elution from a cation exchange column compared to wild-type RNase I, as was reported for RNase M. Likewise, as shown in Table 2, the loss of one arginine and the substitution of several other amino acids would result in a somewhat altered predicted tryptic peptide map. As a consequence, the RNase I from strain MRE600 would be expected to have two peptides (peptides 4 and 5), and perhaps a third peptide depending on separation conditions (peptide 1), that differ from those in wild-type RNase I. At the same time, several of the peptides from wild-type RNase I would be absent from the RNase I of MRE600 (peptides 2, 3, and 5). These tryptic peptide differences between the two RNase I proteins are exactly those reported as the basis to distinguish RNase M and RNase I (Meador et al., 1990). These initial data support the conclusion that RNase M is simply a mutant form of RNase I that is present in strain MRE600.

TABLE 1. Comparison of *rna* sequences from strains CA265 and MRE600.[a]

Nucleotide position[b]	Nucleotide		Amino acid	
	CA265	MRE600	CA265	MRE600
15	G	A	Trp	Stop
70	T	G	Leu	Val
344	G	A	Arg	His
354	A	G	Arg	Arg
378	A	G	Gly	Gly
756	T	A	Pro	Pro
765	T	C	Gly	Gly
797	C	T	Ala	Val

[a]Sequences were determined from 249 nt upstream of the coding region to 83 nt downstream. Only differences within the coding region are shown.
[b]Position 1 is the first nucleotide of the *rna* coding region.

TABLE 2. Predicted tryptic peptides derived from RNase I of CA265 and MRE600.

Peptide[a]	CA265		MRE600	
	Residues	Mass (Da)	Residues	Mass (Da)
1	7–29	2,283	7–29	2,269
2	101–115	1,642		
3	116–118	333		
4			101–118	1,937
5	266–268	310	266–268	336

[a]Tryptic peptides were derived assuming cleavage at every arg and lys residue except in one case in which a pro follows an arg residue. Only those peptides that differed between CA265 and MRE600 are shown. CA265 would be expected to have 34 tryptic peptides and MRE600 would have 33.

Southern analysis of *rna* genes from strains CA265, MRE600, and CF881

As noted earlier, analysis of the sequenced *E. coli* genome gave no indication of a second gene related to *rna* that could be responsible for encoding RNase M. Inasmuch as the sequence of the genome of strain MRE600 had not been determined, the possibility remained that a second gene related to *rna* was present in this bacterium. To eliminate this possibility, southern analysis was performed on chromosomal DNA from strains CA265, MRE600, and CF881 (Fig. 1). DNA from each strain was digested with three restriction enzymes, *Kpn*I, *Xho*I, and *Dra*I, and the separated DNA fragments were hybridized with a probe consisting of

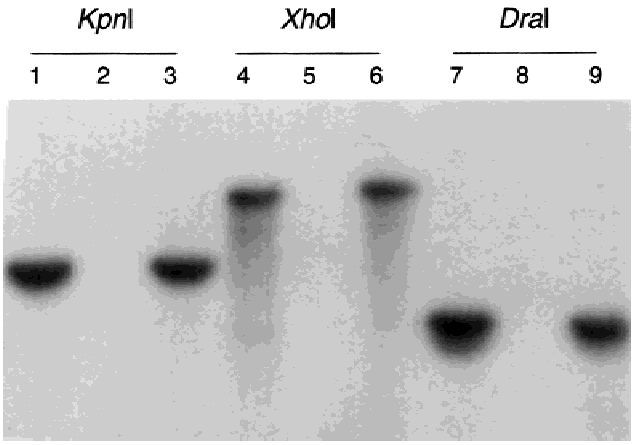

FIGURE 1. Southern analysis of the *rna* gene from strains CA265, CF881, and MRE600. Procedures were carried out as described in Materials and Methods using the three indicated restriction enzymes. CA265: lanes 1, 4, and 7; CF881: lanes 2, 5, and 8; MRE600: lanes 3, 6, and 9.

the full-length *rna* gene. As can be seen in Figure 1, a single band was detected in strain CA265 with each of the restriction enzymes (lanes 1, 4, and 7), and a band of the same size was observed in each case with strain MRE600 (lanes 3, 6, and 9). As a control, no signal was detected in the RNase I deletion strain CF881, with any of the restriction enzymes (Fig. 1, lanes 2, 5, and 8). These data show that strain MRE600 has only a single *rna*-type gene, and that, therefore, RNase M could not be the product of a closely related gene.

RNase I and RNase M activities in strain MRE600

If the RNase M activity isolated from strain MRE600 were due to RNase I, as suggested here, we would expect that residual RNase I activity would be present in the mutant strain. To test this point, RNase I was assayed in strains CA265, MRE600, and CF881 (Fig. 2). Based on these assays, strain MRE600 retains 1% of the RNase I activity present in the wild-type strain, and a level clearly elevated from that in the RNase I deletion strain. Thus, the RNase M activity from strain MRE600 could be a consequence of the residual RNase I.

To further show a relation between RNase M and RNase I, we assayed RNase M, using 5S RNA as substrate, as described by Cannistraro and Kennel (1989). As shown in Table 3, approximately 25% of 5S RNA was degraded in strain MRE600 (based on strain CF881 set to 100) both in the absence of 1 mM EDTA, the condition used by Cannistraro and Kennell (1989), or in its presence, the assay condition used for RNase I. Strain CA265, which has more RNase I activity (Fig. 2), also displays more RNase M activity, as judged by this assay. Moreover, 10 mM $MgCl_2$, which inhibits RNase I, also inhibits RNase M. Although not very quantitative, these data show a strong correlation between RNase M activity, as defined by Cannistraro and Kennell (1989), and RNase I, and support the conclusion that RNase M is due to the residual RNase I in strain MRE600.

DISCUSSION

Taken together, the data reported here strongly support the conclusion that RNase M is a mutated form of RNase I present in *E. coli* strain MRE600, and that it is not a distinct enzyme. Earlier work, repeated here, demonstrating that RNase I accounts for >99% of the EDTA-dependent, nonspecific endoribonuclease activity in extracts (Zhu et al., 1990) had already raised questions about the identity of RNase M, and this concern was heightened when computer analysis of the *E. coli* ge-

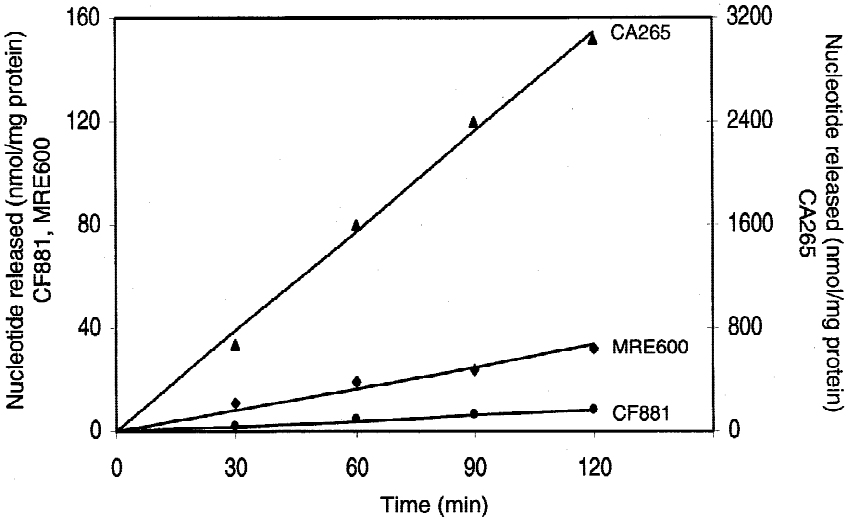

FIGURE 2. Comparison of RNase I activity in strains CA265, MRE600, and CF881. Extracts were prepared and assayed for RNase I activity as described in Materials and Methods. Extracts were assayed for the indicated times using 2.5 μg of protein for CA265 (▲–▲), or 200 μg of protein for MRE600 (♦–♦) and CF881 (●–●). Note the different scale for CA265 compared to MRE600 and CF881.

TABLE 3. Comparison of RNase M activities in strains CA265, MRE600, and CF881.[a]

Extract	Percent 5S RNA remaining[b]		
	No addition	+1 mM EDTA	+10 mM $MgCl_2$
CA265	43	41	101
MRE600	75	65	108
CF881	100	100	100

[a]Assays were carried out as described in Materials and Methods. Data presented are the averages of four experiments.
[b]The 5S RNA remaining after treatment with the CF881 extract was set at 100%.

nome failed to detect any homologs of the *rna* gene (Y. Zuo & M.P. Deutscher, unpubl. observations). The experiments presented also provide a satisfactory explanation for at least some of the properties attributed to RNase M, such as its difference in charge and altered tryptic peptide map. However, further work on RNase I structure and mechanism of action will be needed to explain how the multiple amino acid substitutions in the RNase I of MRE600 lead to the observed changes in its heat stability, subcellular localization, and substrate specificity. Nevertheless, this work emphasizes the importance of using genetic analysis, in addition to biochemical studies, when characterizing newly discovered enzyme activities. In this case, even extensive purification of the relevant protein failed to prevent an erroneous identification.

One interesting finding that arose as a consequence of sequencing the *rna* gene of strain MRE600 was the presence of a UGA termination codon at the position of the fifth amino acid. Because MRE600 retains 1% of the wild-type level of RNase I activity, it is likely that significant readthrough of this codon occurs. UGA codons are known to be particularly leaky, in some cases allowing readthrough at a frequency as high as 10^{-2}, by wild-type $tRNA^{Trp}$ (reviewed in Parker, 1989). Although additional work would be necessary to conclusively eliminate the presence of a UGA suppressor in strain MRE600, we suspect that the low level of residual RNase I activity is simply due to normal readthrough of the UGA codon.

The work described here also has implications for the identity of a number of other endoribonucleases reported to exist in *E. coli*. In particular, we predict that RNase IV (Spahr & Gesteland, 1968) and RNase F (Gurevitz et al., 1982) will also turn out to be manifestations of RNase I. Like RNase I, both enzymes are active in the absence of divalent cations and generate 3′ phosphoryl groups, and RNase IV also has a molecular mass (31 kDa) close to that of RNase I. Although both RNase IV and RNase F were reported to differ somewhat from RNase I based on substrate specificity, and while RNase IV was isolated from an RNase I^- strain, we have already seen here that these are insufficient criteria to characterize these activities as distinct enzymes. Thus, we think there is sufficient reason to propose that these enzymes also are related to RNase I. It will be of interest to see whether future work bears out this idea.

MATERIALS AND METHODS

Bacterial strains

E. coli K-12 (λ) strain CA265 (*lacZ56, trp49, relAl, spoT1, PO1, supF66*; Bachmann, 1996) was considered wild type for these experiments. MRE600 (Wade & Robinson, 1966) is a natural RNase I- deficient strain that was used as the source for purification of RNase M (Cannistraro & Kennell, 1989). It was kindly provided by Dr. R. Wagner, Institute of Physikalische Biologie, Dusseldorf, Germany. Strain CF881 (*recB, xthA, rnaI*) lacks the complete *rna* gene (Zhu et al., 1990).

Culture conditions

Cells were routinely grown at 37 °C in yeast-tryptone (YT) medium or on YT plates.

Chemicals and enzymes

[^{32}P]-phosphate for preparation of radioactive tRNA, [α-^{32}P]dATP for labeling of hybridization probes, and [γ-^{32}P]ATP for end labeling of 5S RNA were obtained from DuPont/NEN. All other chemicals were reagent grade. Restriction enzymes and phage T4 polynucleotide kinase were purchased from New England Biolabs. Oligonucleotides for PCR, DNA sequencing, and RNase M assays were prepared in the University of Miami School of Medicine Department of Biochemistry and Molecular Biology DNA facility.

Synthesis of the *rna* genes of CA265 and MRE600

The *rna* genes from strains CA265 and MRE600 were synthesized by PCR using a forward primer, F1 (5′-GGTCCTGGGGTGATTATTTAC-3′), located 249 nt upstream of the *rna* gene and a reverse primer, R1 (5′-GATGATACTG ACTGTTGCTCA-3′), located 83 nt downstream of the stop codon of the *rna* gene, and the Expand High Fidelity PCR System (Boehringer Mannheim). PCR fragments (expected length 1,172 bp) were gel purified using the Qiaex II Gel Extraction Kit (Qiagen).

Sequencing of the *rna* genes

Gel-purified PCR products from several independent reactions were combined as the templates for sequencing. Primers F1 and R1, and internal primers F2 (5′-CCAGGAT TGCCTAAATCGGTT-3′), complementary to residues 175–195, and R2 (5′-ACTTAGTTTAGCGGCCGTTTC-3′), complementary to residues 339–319, were used to complete the sequences in both directions. Sequencing was carried out in the University of Miami School of Medicine Department of

Biochemistry and Molecular Biology DNA facility. Sequences were assembled and analyzed using Gene Tool Lite (Double Twist, Inc.).

Southern hybridization of *rna* genes

Genomic DNA was isolated from strains CA265 (wild type), MRE600, and CF881 (*rna* deletion) (Wilson, 1994). Ten micrograms of each DNA preparation was digested with the restriction enzymes *Dra*I, *Kpn*I, and *Xho*I, and the fragments separated by electrophoresis on 0.7% agarose. After transfer to a Nylon membrane (Pall Gelman) by standard procedures (Sambrook et al., 1989), hybridization was carried out with the full-length PCR product of the *rna* gene labeled with the Prime-a-Gene Labeling Kit from Promega.

Preparation of radioactive tRNA

[^{32}P]tRNA, used as a substrate for RNase I assays, was prepared from CA265 cells carrying a mutation in the *rna* gene. Cells were grown overnight at 37 °C in YT medium supplemented with [^{32}P]-orthophosphate. After harvesting, cells were washed once with 0.9% saline and tRNA was isolated as described (Deutscher & Hilderman, 1974).

RNase I assay

Cells were grown in YT medium to an $A_{550} \approx 1$. After harvesting, cells were suspended in 20 mM Tris-Cl, pH 7.5, sonicated on ice three times for 20 s each in a Microson Ultrasonic Cell Disrupter at a setting of 4. Samples were centrifuged at 10,000 rpm for 10 min at 4 °C and the resultant supernatant fluid was assayed for RNase I activity. The assay mixture contained 20 mM Tris-Cl, pH 7.5, 1 mM EDTA, 8 μg of [^{32}P]-tRNA ($\approx$10,000 cpm) and 2.5 to 200 μg of supernatant fraction in a total volume of 50 μL. After incubation at 37 °C, the reaction was stopped by addition of 350 μL of cold 0.5% total yeast RNA and 400 μL of 20% trichloracetic acid. After 10 min on ice, the sample was centrifuged at 10,000 rpm for 10 min and 400 μL of the supernatant fraction was removed for determination of acid-soluble radioactivity.

RNase M assay

The RNase M assay was adapted from that of Cannistraro and Kennell (1989) with the following modifications: A mixture of 5S rRNA and tRNA was purified from *E. coli* and used as the substrate. Reaction mixtures contained 10 mM Tris-Cl, pH 7.5, 10 μg of substrate, and 5 μg of supernatant fraction in a total volume of 10 μL. Samples were incubated at 37 °C for 10 min, followed by chilling on ice, extraction with chloroform/isoamyl alcohol, and precipitation with ethanol. One-tenth volume of loading buffer (96% formamide, 20 mM EDTA containing bromphenol blue and xylene cyanol) was added, and the mixture was loaded on a 6% acrylamide gel containing 6 M urea. The gel was preelectrophoresed for 30 min at 20 mA. The sample was subject to electrophoresis at 40 mA until the bromphenol blue ran out of the gel. RNA was electroblotted onto a Biodyne B membrane (Pall Gelman) in 1× TBE buffer at 300 mA for 1 h. At the end of the transfer, the membrane was washed twice with 1× TBE, blocked with ExpressHyb Hybridization solution (Clontech) at 37 °C for 30 min. Ten milliliters of a solution containing a [^{32}P]-end-labeled probe ($\approx 7 \times 10^6$ cpm/mL), complementary to nucleotides 26–47 of 5S rRNA, was added to the mixture and incubation at 37 °C was continued for 1 h. The membrane was then washed with 2× SSC, 0.05% SDS for 40 min with several changes followed by two washes for 20 min each in 0.1× SSC, 0.1% SDS. The membrane was then exposed to a Phosphorimager and the loss of radioactivity in the 5S rRNA region was quantitated using Image Quant software (Molecular Dynamics).

Protein assay

Protein concentration was determined by the method of Bradford (1976).

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