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Escherichia coli rna Gene Encoding RNase I: Cloning, Overexpression, Subcellular Distribution of the Enzyme, and Use of an *rna* Deletion To Identify Additional RNases

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The cloning and overexpression of the *Escherichia coli rna* gene encoding RNase I are described. Only a single copy of the *rna* gene is present on the *E. coli* chromosome. Although cells with as much as a 100-fold increase in RNase I activity were constructed, little effect on cell growth was observed. Overexpressed RNase I was found in the periplasmic space to the same degree (~85%) as wild-type enzyme, suggesting no limitation in RNase I transport. The *rna* clone was used to identify a deletion strain totally lacking the *rna* gene. The normal growth of this strain showed that RNase I is not essential for cell viability. Extracts from the RNase I deletion strain still retained a low level of RNase activity in the presence of EDTA, conclusively demonstrating the existence of additional EDTA-active RNases in *E. coli*. The possibility of a RNase I inhibitor is also discussed.

Escherichia coli RNase I is a low-molecular-weight (M_r, ~25,000), nonspecific endoribonuclease that hydrolyzes RNA in the absence of divalent cations (see reference 21 for a review). In this latter property, it is one of only a few identified *E. coli* RNases that can function without divalent cations (7); in fact, in crude extracts, ~98% of the RNase activity found in the presence of EDTA is due to this enzyme (25). Though RNase I is often bound to 30S ribosomal subunits in cell extracts (23), it is actually thought to reside in the periplasmic space in vivo (19).

Despite the fact that RNase I was identified 30 years ago (9), its role in RNA metabolism remains unclear. A number of studies have suggested that RNase I may be involved in stable RNA degradation under certain conditions. These include elevated temperature in cells containing an F factor (12), nutrient deprivation (14), and exposure to Hg^{2+} ions during exponential growth (3). RNase I also participates in the degradation of RNA synthesized during relaxed growth conditions (13). Mutant strains deficient in RNase I have been identified (10), but they are viable and show no obvious phenotype. On the other hand, these mutants, which retain about 1% of the wild-type level of activity, have proven extremely useful for many studies of RNA metabolism in which low levels of RNase activity are required, although for some experiments even 1% residual activity may be excessive.

A number of observations related to RNase I remain to be explained. Thus, as noted above, RNase I can degrade stable RNA under certain conditions, but it is not clear how this is accomplished if RNase I is in the periplasmic space and presumably not accessible to the intracellular RNA. Second, several RNase activities similar but not identical to RNase I have been described (1, 2, 19), and all are decreased in RNase I^- mutants (10), suggesting that they are different forms of the same enzyme. The relationship among these various forms of the enzyme is not understood and is complicated by the absence of any information about RNase I structure. Finally, although RNase I^- mutant strains are viable, it is not clear whether the residual 1% of activity is responsible or even whether the residual activity is RNase I.

To approach these questions, we have begun to study the *rna* gene encoding RNase I. In this report, we describe the cloning of the *rna* gene and the use of the cloned gene to overexpress RNase I activity to ascertain its distribution between the periplasmic space and the cytoplasm. We also describe a mutant strain in which the *rna* gene has been totally deleted and the use of extracts from this strain to identify another RNase, active in the presence of EDTA, that is distinct from RNase I.

MATERIALS AND METHODS

Bacterial strains and plasmids. Wild-type *E. coli* K-12 strain CA265 and its multiple RNase-deficient derivative 20-12E/18-11 (RNase I^- II^- D^- BN^- T^-) have been described previously (8). Strain CF881 (*recB xthA rnaI*), obtained from Michael Cashel, and strain JC7623 (*recB recC sbcB*), obtained from Paul Schimmel, were used for linear transformation. Strain UT481 [Δ(*lac-pro*) *hsdS* (r^- m^-) *lacI*^{q} *lacZ*], obtained from Gordon Carmichael, was used for growth of bacteriophage and plasmids. Plasmids pHC79 (11), pBR328 (22), and pUC8 (24) have been described.

Culture conditions. Cells were routinely grown at 37°C in LB or YT medium or on YT plates (18). Glucose, when present, was at 0.4%. Antibiotics were added at the following concentrations: ampicillin, 50 μg/ml; tetracycline, 12.5 μg/ml; and chloramphenicol, 30 μg/ml. Growth in liquid culture was followed by measurement of A_{550}.

Chemicals and enzymes. Restriction endonucleases, T4 DNA ligase, and BAL 31 nuclease were products of New England BioLabs, Inc. Carrier-free $^{32}P_i$ was obtained from Dupont, NEN Research Corp., and [^{3}H]poly(A) was from

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Amersham Corp. [^{32}P]tRNA and [^{32}P]rRNA were prepared from labeled *E. coli* cells as described previously (25).

Preparation of extracts and RNase assays. For routine assays, cells were generally grown to an A_{550} of 1, collected by centrifugation, and suspended in 1/10 volume of 20 mM Tris chloride (pH 7.5). The suspension was sonicated for 30 s and used directly for assay. RNase assays were carried out in 0.1-ml reaction mixtures containing 20 mM Tris chloride (pH 7.5), 1 mM EDTA, 20 μg of [^{32}P]tRNA, and 10 to 50 μl of cell extract. After incubation at 37°C for the indicated period of time, acid-soluble radioactivity was determined as described previously (25). For the experiment presented in Table 2, cells were suspended in one-fifth volume of 10 mM Tris chloride (pH 8.0)–1 mM EDTA and assayed under the same conditions. Sonication was for 45 s in this experiment, and extracts were assayed after centrifugation.

Preparation and screening of a cosmid library. The cosmid library, a gift from P. deBoer, was prepared from a partial *Sau*3A digest of DNA from strain ED51 (F'126 *pro trpA his metE*). Fragments of 45 to 50 kilobases (kb) were size selected, ligated into pHC79, and packaged in vitro. Infection of strain 20-12E/18-11 was carried out under standard conditions, and Amp^r transformants were isolated. Extracts were prepared as described above for screening for elevated RNase activity.

Recombinant DNA and genetic procedures. Standard recombinant DNA techniques described by Maniatis et al. (17) were used throughout. Chromosomal DNA was prepared as described previously (27). Phage P1-mediated transduction was carried out by standard procedures (18), using P1 *vir*.

Subcloning of the *rna* gene. The *rna* gene was subcloned from cosmid 79 by digestion with *Eco*RV to a ~5-kb fragment that was ligated into pBR328. The resulting plasmid was opened at the *Pvu*II site and digested with BAL 31 to inactivate the endogenous chloramphenicol acetyltransferase (*cat*) gene. A *cat* fragment was then placed into the *Sma*I site of the insert to generate pECS-CAT. This plasmid was used for the linear transformation experiments. The fragment containing the *rna* gene was shortened further by digestion with *Eco*RI. The resulting 2-kb fragment was inserted into pBR328 to give plasmid pE4 and into pUC8 to give plasmids pUR1 and pUR2 (two orientations). Cells harboring the latter two plasmids contained about 100 times the wild-type level of RNase activity.

RESULTS

Isolation of the *rna* gene. A cosmid library, containing large fragments of the *E. coli* genome and prepared as described in Materials and Methods, was screened for transformants that contained elevated levels of RNase I activity. To facilitate screening, an *E. coli* strain, 20-12E/18-11, deficient in multiple RNase activities, including RNase I, was used as the recipient for cosmid infection, and transformants were combined in groups of five for RNase assays (26). Of 400 transformants screened in this fashion, one clone, no. 79, was identified that displayed elevated activity in the presence of EDTA against a variety of RNA substrates [tRNA, rRNA, and poly(A)] when compared with the parental strain, 20-12E/18-11, and the wild-type RNase$^+$ parent, CA265. With tRNA as a substrate, the RNase activity in clone 79 was elevated about 20-fold over wild-type levels and about 400-fold over the levels in the RNase-deficient strain (Table 1). Elevated RNase activity was observed when cells were ruptured by sonication (Table 1) or French press or when permeabilized with Tris-EDTA (data not shown). Likewise, similar levels of elevated activity were found when cells were opened by using a variety of buffer conditions (Table 1).

TABLE 1. RNase activity of clone 79[a]

Strain	Description	RNase sp act (nmol/h per mg of protein)
CA265	Wild type	600–1,200
20-12E/18-11	RNase I$^-$	10–50
Clone 79	Transformant of 20-12/18-11	13,000–18,000

[a] Cells were grown, ruptured by sonication, and assayed as described in Materials and Methods. The data presented are the results of four experiments using the following different buffer conditions for extraction of cells: 20 mM Tris chloride (pH 7.5), 10 mM Tris chloride (pH 7.5), 20 mM glycine-NaOH (pH 8.9), or 10 mM Tris chloride (pH 7.5) containing 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride.

The finding that the elevated RNase displayed no substrate specificity and that it was active in the presence of EDTA strongly supports the idea that RNase I was elevated in clone 79. This was confirmed by gel filtration on Ultrogel AcA44 of S-100 fractions from clone 79, which indicated that the elevated activity was due to a protein of 25,000 to 30,000 daltons, the apparent size of RNase I (6).

To prove conclusively that the cloned gene leading to elevated RNase activity was, in fact, *rna* encoding RNase I, cosmid 79 was subcloned as described in Materials and Methods. One subclone, pECS-CAT, contained a ~5-kb fragment from clone 79 into which a 1.3-kb piece of DNA encoding the *cat* gene from pBR325 was inserted at a *Sma*I site. Cells transformed with this plasmid became chloramphenicol resistant but displayed the usual amount of elevated RNase activity, indicating that the inserted *cat* gene was functional and adjacent to the RNase gene of interest. This plasmid was then linearized with *Bam*HI and used to transform strain JC7623 in order to place the *cat* gene on the chromosome adjacent to the unknown RNase gene.

Three transformants were isolated that were chloramphenicol resistant and ampicillin sensitive, suggesting that they had integrated the insert from pECS-CAT into the chromosome. To confirm this, chromosomal DNA was isolated from the three transformants and the parental strain, JC7623, and analyzed by Southern blotting using a *cat* gene probe (Fig. 1A) and an RNase gene probe (Fig. 1B). Chromosomal DNA from all four strains displayed a band that hybridized to the RNase gene probe and differed in size from the plasmid (Fig. 1B). In contrast, only DNA from the transformants hybridized to the *cat* probe, whereas parental DNA did not (Fig. 1A), as expected if the *cat* gene had been integrated. It was also noted that the band corresponding to transformant 2 differed from the other two transformants in either position (Fig. 1A) or intensity (Fig. 1B). Further analysis of this transformant indicated that it had integrated a second copy of the RNase gene. This was shown by the presence of two bands on the Southern blot after *Bgl*II digestion (Fig. 1C) and by twice the level of RNase activity in extracts of transformant 2 compared with transformants 1 and 3 and the parental strain (data not shown).

Phage P1 lysates prepared from each of the transformants were then used to transduce strain 20-12E/18-11 (RNase I$^-$) to chloramphenicol resistance. Of eight chloramphenicol-resistant transductants picked, extracts from six were found to contain RNase I activity, with the highest activity in transductants derived from transformant 2 (Table 2). In a

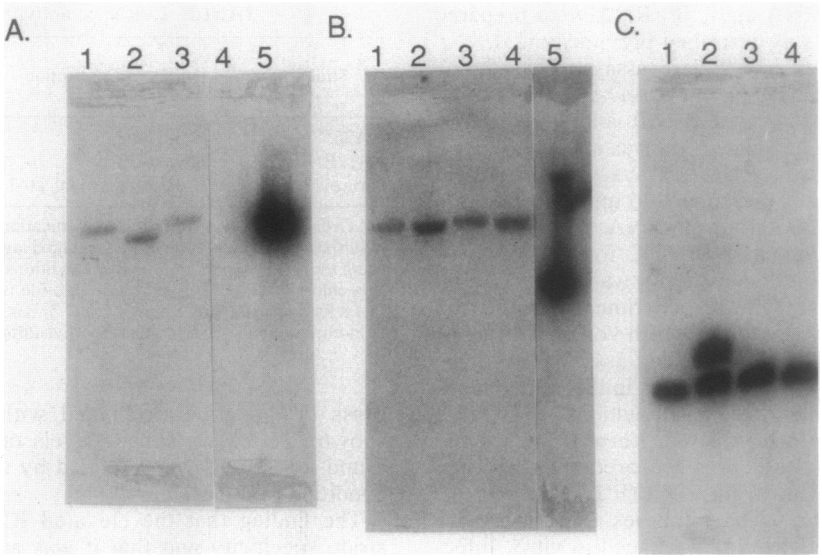

FIG. 1. Southern hybridization of linear transformants of stain JC7623. Chromosomal DNA from each of the transformants and the parental strain was digested with either *Eco*RV (A and B) or *Bgl*II (C), run on a 0.8% agarose gel, and hybridized with a labeled *cat* fragment (A) or plasmid pE4 (B and C). (A) Lanes: 1 to 3, transformants 1 to 3; 4, JC7623; 5, plasmid pECS-CAT. (B) Lanes: 1 to 3, transformants 1 to 3; 4, JC7623; 5, plasmid pE4. (C) Lanes: 1 to 3, transformants 1 to 3; 4, JC7623.

second experiment, the P1 lysate from transformant 3 was used to transduce another RNase I$^-$ strain, CF881. Of 38 chloramphenicol-resistant transductants isolated, at least 37 regained RNase I activity. These data demonstrate that DNA originally derived from clone 79, when integrated into the chromosome in single copy, can correct the RNase I$^-$ defect of various mutant strains, demonstrating that the clone carries the *rna* gene.

Identification of an *rna* deletion strain. We initially attempted to carry out linear transformation of pECS-CAT by using strain CF881. Since this strain is RNase I$^-$ and proficient for linear transformation (27), it would have enabled us to identify the unknown RNase gene as *rna* in a single step. Surprisingly, although other genes could be integrated into the chromosome of CF881 by linear transformation, this could not be accomplished with the *rna* gene.

TABLE 2. RNase I activity of chloramphenicol-resistant transductants[a]

Strain[b]	RNase activity (nmol/10 min)
Transformant 3	0.70
Transductants	
1-A	1.06
1-B	1.63
2-A	2.21
2-B	1.84
3-A	0.18
3-B	0.91
3-C	0.99
3-D	0.02
20-12E/18-11	0.06

[a] Phage P1 lysates from linear transformants of strain JC7623 were used to transduce the RNase I$^-$ strain 20-12E/18-11. Several transductants were picked from each cross and assayed for RNase I activity as described in Materials and Methods. The activities of one of the donor strains (transformant 3) and the recipient strain are also shown. Assays were for 10 min at 37°C. Activities of all extracts were normalized to the same amount of protein.

[b] Transductants beginning with numbers 1 to 3 came from P1 lysates prepared from transformants 1 to 3, respectively.

Further analysis of chromosomal DNA from CF881 by dot blotting (not shown) and Southern hybridization (Fig. 2) revealed that this cell lacked the *rna* gene. The fact that no hybridization at all was found with the *rna* probe indicates that the complete *rna* gene is missing in strain CF881. However, the full extent of the deletion is not known.

In the construction of strain CF881, a Tn*10* transposon originally near the mutant *rna-19* gene was eliminated by growth in fusaric acid (16) to make the strain tetracycline sensitive (M. Cashel, personal communication). Since re-

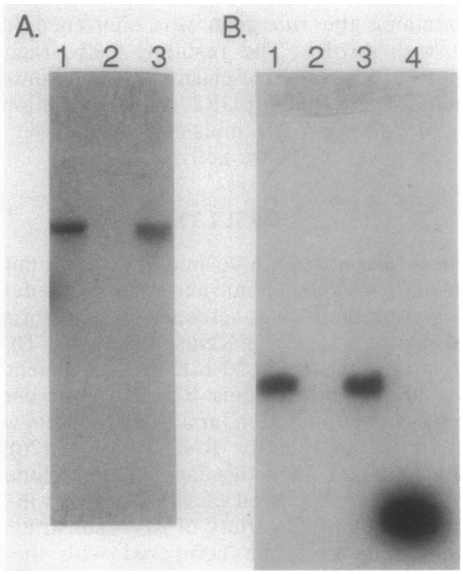

FIG. 2. Southern hybridization of DNA from strains 20-12E/18-11, CF881, and JC7623 probed with the *rna* gene. Chromosomal DNA from each of the strains was digested with either *Eco*RV (A) or *Bgl*II (B), run on a 0.8% agarose gel, and hybridized with labeled pE4. (A) Lanes: 1, 20-12E/18-11; 2, CF881; 3, JC7623. (B) Lanes: 1, 20-12E/18-11; 2, CF881; 3, JC7623; 4, plasmid pE4.

TABLE 3. Subcellular distribution of RNase I in wild-type and overexpressing cells[a]

Strain	RNase I activity				
	Total cell (nmol)	Spheroplast		Periplasm	
		nmol	%	nmol	%
Expt 1					
CA265	2.80	0.34	12	2.46	88
79 in 20-12E/18-11	36.8	4.32	16	22.5	84
Expt 2					
CA265	1.39	0.17	11	1.34	89
pUR1 in 20-12E/18-11	ND	10.8	17	53.4	83

[a] Cells were grown, ruptured by sonication, and assayed as described in Materials and Methods. Portions of 5 ml were used for total cell activity, and 10-ml portions of the same cultures were used for spheroplast and periplasm preparation by the procedure of Neu and Heppel (19). Spheroplasts were opened by sonication. Portions of each fraction (total, spheroplast, and periplasm) were assayed for RNase I activity, using 10 μg of [^{32}P]tRNA (200 to 500 cpm/nmol). Incubation was for 10 min at 37°C. Activities of all fractions were normalized to the same volume of extract. ND, Not determined.

moval of Tn*10* is known to promote nearby deletions (15), the *rna* gene was undoubtedly removed at this step. However, the absence of the *rna* gene was not detected because the strain was already RNase I$^-$. In contrast, the *rna-19* allele, tested with DNA from strain 20-12E/18-11, gave a positive signal for the *rna* gene (Fig. 2), indicating that the RNase I deficiency of this strain was not due to a deletion of substantial size. In any event, the unexpected identification of an *rna* null mutant provides us with a strain totally devoid of RNase I activity that should prove useful for a variety of studies. These results and those presented in Fig. 1 also indicate that only a single *rna* gene is present on the *E. coli* chromosome.

Despite the total absence of RNase I, strain CF881 grows normally in rich medium. These results indicate that RNase I is not an essential enzyme for *E. coli* under laboratory conditions. On the other hand, strain CF881 still retains a low but easily measurable level of RNase activity in the presence of EDTA, suggesting the existence of another enzyme (see below).

Properties of cells with elevated RNase I. Cells harboring cosmid 79 grow normally despite the fact that they contain as much as 20-fold-higher levels of RNase I than do wild-type cells. In contrast, cells carrying plasmids pUR1 or pUR2 display variable effects. These cells can have as much as a 120-fold elevation of RNase I activity, but in some cycles of growth the increase in activity was lower. In addition, the growth rates of the cells varied in different experiments, although in some cases cells with high elevations of RNase activity grew essentially normally. In all experiments, cells remained ampicillin resistant, indicating that they had not completely lost the plasmid. We do not yet understand the molecular basis for these effects, but it appears that extremely high levels of RNase I can lead to the generation of cell variants in the culture that have lower RNase activity and some growth advantage.

RNase I is located predominantly in the periplasmic space of *E. coli* cells (19). It was of interest to determine whether cells with elevated levels of the enzyme displayed a similar distribution of activity. Accordingly, parental wild-type cells and cells carrying cosmid 79 or plasmid pUR1 were converted to spheroplasts, and the RNase I activity released into the medium and that retained by the spheroplast were measured (Table 3). In wild-type cells, close to 90% of RNase I activity was released from the cells upon spheroplast formation, in good agreement with earlier data (19). Essentially identical distributions were found with cells containing cosmid 79 or pUR1. In addition, the sum of the spheroplast and periplasm activities approximated that of the total cell, indicating that all of the activity can be accounted for. Under these same conditions, about 90% of β-galactosidase activity was retained within the spheroplast (not shown), indicating that release of RNase I was not due to spheroplast lysis during the procedure. These data suggest that the cellular machinery for transporting proteins into the periplasmic space is sufficiently active to handle the increased amount of RNase I present in the overexpressing strains.

Properties of overexpressed RNase I. During the course of these studies, it became apparent that the cloned RNase I activity from overexpressing strains was slightly different from that from the parental wild-type strain. Although the two activities had many identical properties and were temperature resistant, as expected (23), the cloned enzyme was less sensitive to inactivation by incubation with dithiothreitol. In addition, the relative activity of the cloned enzyme on tRNA compared with poly(A) was severalfold lower than the wild-type activity (data not shown). We do not yet know the reason for these subtle differences. Since the experiments were carried out with crude preparations of the enzyme, the differences may be due to factors other than RNase I itself. Purification of the wild-type and cloned RNase I will be necessary to resolve this question.

Identification of RNase activity in the *rna* deletion strain. Currently available RNase I$^-$ strains retain about 1% of wild-type RNase activity assayed in the presence of EDTA (10). In agreement with these earlier data, we found that extracts of strain 20-12E/18-11 had slightly more than 1% of the activity of the RNase$^+$ parental strain, CA265 (Fig. 3). It is not clear whether this activity is due to residual RNase I or to another enzyme. However, strain CF881, in which the *rna* gene was completely deleted, still retained about 40% of the activity of the RNase I$^-$ mutant (Fig. 3). These results indicate that at least one additional EDTA-active RNase is present in *E. coli* and that the residual RNase activity in strain 20-12E/18-11 is probably a combination of some residual RNase I activity and an additional RNase(s).

Analysis of the activity in strain CF881 by gel filtration and glycerol gradient centrifugation revealed several peaks able to hydrolyze [^{32}P]tRNA in the presence of EDTA. Studies are in progress to characterize these activities.

DISCUSSION

This report describes the cloning and overexpression of the *E. coli rna* gene encoding RNase I and the use of this system to answer several important questions about RNase I. First, we have shown that it is possible to overexpress RNase I to quite high levels without deleterious effects on the cell. This result was surprising, since RNase I is a very active, nonspecific nuclease and would have been expected to degrade much of the cellular RNA. One possible explanation for the lack of an effect of the high level of RNase I is that it is not accessible to the RNA. Despite the high level of overexpression, about 85% of the activity was released upon spheroplast formation and presumably was in the periplasmic space (19). Although overexpression of the other 15% of activity associated with the spheroplast should have been sufficient for RNA degradation, it is not certain that even this portion of the activity is within the cell, since it could have

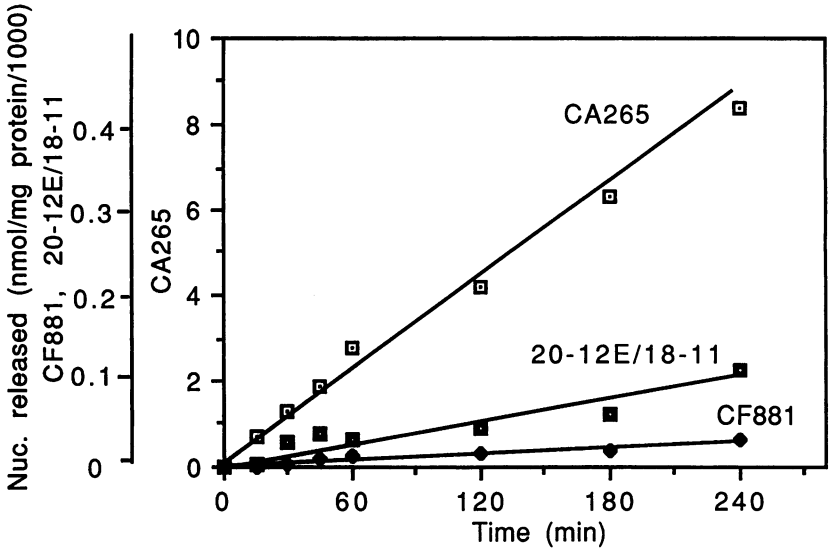

FIG. 3. Comparison of RNase activity in strains CA265, 20-12E/18-11, and CF881. Cells were grown to an A_{600} of 1, concentrated from 20 ml to 0.7 ml in 20 mM glycine-NaOH (pH 8.5), and sonicated with two 20-s pulses. Cell debris was removed by centrifugation, and the supernatant fractions were assayed for RNase activity for the indicated times. Assay mixtures of 100 μl contained 25 mM Tris chloride (pH 8.0), 10 mM EDTA, 27 μg of [^{32}P]tRNA (~10^3 cpm/nmol), and 6 μg of CA265 extract, 22 μg of 20-12E/18-11 extract, or 48 μg of CF881 extract. Acid-soluble radioactivity was determined as described in Materials and Methods. All data were normalized to 1 mg of protein. Note the different scale for CA265 activity.

been associated with the outside of the internal membrane. RNase I is known to associate with the debris fraction of cells (2).

An alternative possibility is that any RNase I within the cell is in an inactive form. It is known that RNase I binds tightly to ribosomes in vitro (6, 23) and may do so in vivo. A Hg-sensitive inhibitor of RNase I has also been suggested (3). One piece of evidence supporting the idea that some sort of inhibitor of RNase I might be present in extracts is our finding that cosmid 79 overexpressed RNase I about 20-fold, even though pHC79 with a large insert should only be present at about five copies per cell (11). In previous screenings of this library for other RNase genes (5, 26), we observed elevations of other RNases of less than fivefold. The apparent 20-fold overexpression in the case of RNase I could be due to the presence of an inhibitor that normally keeps most of the cellular pool of this enzyme in an inactive form but is not present at a sufficient level to inactivate the overexpressed enzyme. Also, the sensitivity of RNase I to dithiothreitol in crude extracts could be due to activation of such a Hg-sensitive, thiol-activated inhibitor, as has been found in eucaryotic cells (20). If such an inhibitor is also present in *E. coli*, it would indicate that RNase I activity can be regulated in vivo and would provide a simpler system than eucaryotes to study such regulation. A regulatable pool of RNase I within the cell would also provide an explanation for some of the undefined physiological effects of RNase I mentioned in the introduction. Clearly, further analysis of this system for a possible inhibitor would be worthwhile.

Probably the most important benefit to come from this work is the identification of an *rna* deletion strain. This strain should prove extremely useful for many studies of RNA metabolism in *E. coli*, since it has even lower activity than the currently available RNase I$^-$ strains (10). RNase I is such an active enzyme that even 1% residual activity has made mRNA isolation and Northern (RNA) blots difficult in *E. coli*. Until now, it has also not been clear whether the small amount of residual activity found in RNase I$^-$ strains is, in fact, due to RNase I. The data presented here demonstrate that one or more additional RNase activities, as well as some residual RNase I, are present in the usual RNase I$^-$ strains. The identity of the additional activity(ies) is not known.

Several other RNases active in the presence of EDTA have been reported. These include RNase IV, RNase F, RNase N (7), and the recently described RNase M (4). None of these activities has been sufficiently characterized to ascertain that they are distinct enzymes. In addition, although each was identified in extracts of RNase I$^-$ strains, the possibility still exists that they are simply mutant forms of RNase I with much less activity and somewhat altered specificity. With the identification of residual activity in the RNase I deletion strain reported here, there is no doubt that RNase I is not the only EDTA-active enzyme present in *E. coli*. Purification studies, now in progress, should clarify how many additional enzymes exist.

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