Retention of Core Catalytic Functions by a Conserved Minimal Ribonuclease E Peptide That Lacks the Domain Required for Tetramer Formation*

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Ribonuclease E (RNase E) is a multifunctional endoribonuclease that has been evolutionarily conserved in both Gram-positive and Gram-negative bacteria. X-ray crystallography and biochemical studies have concluded that the Escherichia coli RNase E protein functions as a homotetramer formed by Zn linkage of dimers within a region extending from amino acid residues 416 through 529 of the 116-kDa protein. Using fragments of RNase E proteins from E. coli and Haemophilus influenzae, we show here that RNase E derivatives that are as short as 395 amino acid residues and that lack the Zn-link region shown previously to be essential for tetramer formation (i.e. amino acid residues 400–415) are catalytically active enzymes that retain the 5’ to 3’ scanning ability and cleavage site specificity characteristic of full-length RNase E and that also confer colony forming ability on rne null mutant bacteria. Further truncation leads to loss of these properties. Our results, which identify a minimal catalytically active RNase E sequence, indicate that contrary to current models, a tetrameric quaternary structure is not required for RNase E to carry out its core enzymatic functions.

In Escherichia coli, endoribonuclease E (RNase E) carries out a wide variety of functions, including the processing of 9S ribosomal RNA (1, 2), the degradation of bulk RNA (3–7), the degradation or processing of a wide variety of messenger and structural RNAs (8–10) (for recent review, see Refs. 11 and 12), the control of plasmid DNA replication (13), and the removal of poly(A) tails from transcripts (14, 15). The 118-kDa E. coli RNase E protein, which is encoded by the rne gene (16), contains three distinct regions: an amino-terminal domain that encodes the catalytic activity (17, 18), a centrally located arginine-rich segment that has strong RNA binding activity (17, 19), and a carboxyl-terminal region that serves as a scaffold for the binding of other proteins that are assembled into a ribonuclease-lytic complex known as the degradosome (20–23). A closely related endoribonuclease, RNase G (previously known as the MreE or CafA protein) (9, 24, 25), consists almost entirely of sequences showing homology to the catalytic domain of RNase E (Fig. 1A).

The RNase E/G family of proteins has been classified into four subgroups according to the position of the highly conserved catalytic domain (26). Sequence analysis indicates that the genomes of E. coli and many other Proteobacteria encode both a type I RNase E/G enzyme, typically containing 900–1100 amino acid residues and having the ~500-residue catalytic domain at the amino-terminal end, and a type IV family member (i.e. RNase G), consisting almost entirely of sequences conserved between the two enzymes in an ~500-amino acid residue protein. However, other bacterial genomes encode only one RNase E/G homolog (26, 27). Although all members of the RNase E/G family that have been experimentally studied cleave their substrates in A+U-rich regions, the substrate sites cleaved by RNase G and RNase E are not identical (9, 25). Moreover, despite extensive homology between the catalytic domains of all members of the RNase E/G family, biochemical studies indicate that the RNase E of E. coli can degrade substrates quasi-processively in a 3’ to 5’ scanning mode, whereas RNase G from the same organism has a distributive mode of action (28).

The evolutionarily conserved region of members of the RNase E/G family of enzymes is ~500 amino acids in length, and a 498-residue amino-terminal RNase E fragment of the E. coli RNase E protein retains endoribonucleolytic activity in vitro (17). Bacteria expressing a shorter fragment consisting of residues 1–427 fused with the last 25 residues of the protein are reported to be viable, indicating that the conserved fragment is not required in its entirety for bacterial viability. However, these bacteria are less normal in mRNA degradation than a temperature-sensitive rne mutant at 37 °C and are nonviable at 44 °C (29). Recently, the crystal structure of the catalytic domain of RNase E was solved using a fragment consisting of amino acid residues 1–529 (30). It was shown that the 529-amino acid segment contains two domains that are joined by a Zn-link (amino acids 400–415). The Zn-link is required for RNase E to form a homotetrameric quaternary structure, and the formation of such tetramers has been thought to be required for the proper functioning of the enzyme (30, 31).
The investigations reported here were aimed at defining the minimal fragment of *E. coli* RNase E that mediates various known functions of this endoribonuclease and elucidating the structural components needed for these functions. Starting with the well studied 498-residue amino-terminal fragment of *E. coli* RNase E (N-Rne), we constructed a series of *rne* gene deletions that truncate the protein from either end. Here we show that a peptide that extends 395 or 415 amino acids from the amino-terminal end of the RNase E proteins of *E. coli* and *Haemophilus influenzae* is (despite the lack of the Zn-link and the small homodimer-forming domain shown previously to be required for tetramer formation) sufficient for ribonuclease activity, cleavage site specificity, and 3’–5’ scanning of substrate *in vitro*, as well as for *in vivo* complementation of an *rne* null mutation. Our finding that such functions are mediated by these truncated proteins indicates that, contrary to current models, a tetrameric quaternary structure is not essential for the core enzymatic functions of RNase E.

**EXPERIMENTAL PROCEDURES**

*Plasmids and Bacterial Strains*—A plasmid encoding an *E. coli* N-Rne fragment consisting of amino acid residues 1–498 followed by a hexahistidine affinity tag and a thrombin cleavage site in a pET16b vector (Novagen) has been described (17, 32). To construct a plasmid expressing a fragment of *H. influenzae* RNase E, the coding sequence for residues 16 to 476, as well as a hexahistidine affinity tag and a thrombin cleavage site specificity, and 3’ to 5’ scanning of substrate *in vitro*, as well as for *in vivo* complementation of an *rne* null mutation. Our finding that such functions are mediated by these truncated proteins indicates that, contrary to current models, a tetrameric quaternary structure is not essential for the core enzymatic functions of RNase E.

*RNase E Minimal Catalytic Domain*
In Vivo Complementation Assays—The Ap<sup>+</sup> pRNG3-based plasmids into which segments coding for fragments of RNase E or RNase G proteins had been inserted were introduced by transformation into E. coli strain KSL2000 to test their ability to complement an rne deletion using methods described previously (10). Briefly, the rne null mutation of KSL2000 was initially complemented by a Km<sup>+</sup> plasmid carrying the gene for full-length E. coli RNase E under the control of an arabinose promoter. After transformation with an Amp<sup>+</sup> plasmid encoding an RNase E or G fragment for which transcription was initiated at an IPTG-inducible lacUV5 promoter, cells were cultured on a LB plate containing 0.1% arabinose, 50 μg/ml ampicillin, and 30 μg/ml kanamycin. The colonies picked from the LB plates were then inoculated into LB medium containing the same concentrations of arabinose, ampicillin, and kanamycin and grown to an optical density at 600 nm of 0.6. At that cell density, 10 μl of 1:10<sup>3</sup> to 1:10<sup>6</sup> dilutions of bacteria were spotted onto LB plates containing 50 μg/ml ampicillin and amounts of IPTG (1, 10, 100, or 1000 μM) sufficient to induce expression of the truncated RNase E/G peptide being tested (12).

RESULTS

Expression and in Vitro Characterization of Stable Fragments of RNase E and RNase G—N-Rne has been employed previously for investigation of substrate specificity (14, 17), mode of action (28), and inhibition by other cellular proteins (34). To identify the minimal domain that retained activity, additional constructs for expression of N-Rne fragments with deletions from either the amino or the carboxyl terminus were made (Fig. 1B) utilizing an intein-based expression and affinity purification methods (see “Experimental Procedures”). RNase E was predicted from its amino acid sequence to have an RNA-binding S1 domain (Pfam PF00575) near the amino terminus, spanning residues 35 to 119 (16, 35). Constructs designed to produce a series of RNase E deletion variants lacking amino acid residues through the S1 domain were generated; specifically, residues 1–115, 1–129, and 1–163 were deleted in independent constructs. However, the protein fragments encoded by these constructs showed very low solubility and were found to form inclusion bodies when overexpressed in E. coli.

Systematic deletions from the carboxyl terminus of N-Rne were made. A fragment consisting of residues 1–494 showed less aggregation than the His<sub>6</sub>-tagged 1–498 N-Rne fragment but also formed hetero-oligomers on gel filtration and failed to form crystals. Using a coarse deletion grid of 20 residues, we identified a subset of the constructs used in this work. +, apparent full activity; (+), significantly attenuated activity; −, no measurable activity under the conditions tested.

![Diagram of RNase E Minimal Catalytic Domain](image)

**FIGURE 1. RNase E/G fragments.** A, diagram showing the relative sizes and conserved domains in the full-length (FL) RNase E and RNase G proteins. B, summary of a subset of the constructs used in this work. +, apparent full activity; (+), significantly attenuated activity; −, no measurable activity under the conditions tested.

**FIGURE 2. Endoribonuclease cleavage assay of N-terminal fragments of RNase E and its homologs.** A, the sequence of the oligoribonucleotide substrate, BR30M contains three 10-nucleotide repeats of sequence from the 5′ single-stranded region of the natural substrate RNA I (28), with 2′-O-methyl modification of two nucleotides flanking the cleavage site of the second repeat (underlined). The sites of RNase E/G cleavage are indicated by a solid arrow; the open arrows indicate the sites that can be cleaved only by RNase G, which cleaves substrates in distributive mode with no directionality. The solid triangle indicates the site that is not cleaved, because of 2′-O-methylation. B, gel showing migration of the 32P-labeled BR30M substrate and its cleavage products. Equal amounts of labeled substrate and 50 ng of each protein were used in the assay, and aliquots were taken at the indicated time points. For RNase E derivatives having quasi-processive activity, only the site at the 3′ end from modified nucleotides can be cleaved, as the enzyme cannot cleave and progress beyond the modified site. This cleavage yields a major product of 25 nt. C, cleavage by fragments of RNase E homologs. The BR30M RNA substrate and 50 ng of protein were used for each assay. Only E. coli RNase G, which cleaves substrate randomly without directionality, can cleave BR30M at the 5′ end from the modified nucleotides (5 and 6 nt) (25, 28).
RNase E cleavage specificity and mode of action (28). BR30M contains three repeats of a sequence that normally is susceptible to RNase E cleavage. However, in BR30M, there are 2'-O-methyl-modified nucleotides in the central repeat, preventing cleavage at that site. During quasi-processive RNase E scanning of BR30M from the 3' to 5' direction, transit of the endoribonuclease past the 2'-O-methyl-modified site is blocked, and only the cleavable sequence 3' to the modified site is attacked, generating a 25-nt 5'-labeled cleavage fragment (28). We found that RNase E fragments as short as 395 residues showed the ability to generate the expected 25-nt fragment, albeit less well than the longer RNase E-derived peptides that we tested (Fig. 2B). A 300-residue peptide was devoid of detectable activity (Fig. 2B), as was one that was only five residues shorter than Rne 395 (data not shown). These findings indicate that truncations that result in RNase E peptides as short as Rne 395 retain the quasi-processive 3' to 5' scanning mode of action and cleavage site specificity that is intrinsic to E. coli Rne proteins containing the Zn-link region.

As expected from earlier results, (30, 36) the Rne 395-truncated E. coli RNase E peptide did not detectably form tetramers, as determined by gel filtration chromatography using the same buffer conditions employed for cleavage assays (Fig. 3). Moreover, rather than showing the dimeric higher order structure reported for larger fragments of the RNase E protein (31, 37) Rne 395 was eluted from gel filtration columns predominantly as monomers (Fig. 3).

Fragments of H. influenzae RNase E (residues 16 to 399; equivalent to E. coli Rne 400 and 85% identical in amino acid sequence in the overlapping region), E. coli RNase G (residues 1–400; E. coli Rng 400), and H. influenzae RNase G (residues 1–396; 36% identical to E. coli RNase E and 71% identical to E. coli RNase G) were expressed, purified, and characterized. All of these truncation fragments lacked the enzyme segment containing the Zn-link region required for tetramer formation by E. coli RNase E. As seen in Fig. 2B, the truncated 16 to 399 H. influenzae RNase E fragment showed the same cleavage site specificity and mode of action as a longer fragment, 16 to 476, which contains the Zn-link, and was even more active than E. coli Rne 494, yielding an additional cleavage at the adventitious site found earlier to be cut by highly active longer length RNase E derivatives (28). Equivalent minimal length fragments of H. influenzae Rng (1–396) (Fig. 2B) and E. coli Rng (data not shown) did not show enzymatic activity on this substrate.

In Vivo Complementation of an rne Null Mutation in E. coli by RNase E and RNase G Fragments — The retention of in vitro endoribonuclease activity of truncated RNase E and G fragments prompted us to test their in vivo activity as assayed by their ability to confer colony forming ability on an E. coli strain carrying an rne null mutation. Using methods described previously (10), plasmid-encoded ribonuclease proteins were expressed under control of a lacUV5 promoter regulated by IPTG added to the culture medium. The rne null mutation was complemented by E. coli Rne 395 in the presence of 10 μM IPTG, although cell growth was slower (Fig. 4). The H. influenzae RNase E fragment 16 to 476 also complemented the rne null mutation in the presence of 10 μM IPTG. The shorter H. influenzae protein fragment, consisting of residues 16 to 399, conferred colony forming ability only at IPTG concentrations of 100 mM or higher; and cells expressing the RNase G fragments, E. coli Rng 400 and H. influenzae Rng 396, were unable to form colonies even at an IPTG level of 1000 μM. Thus, the in vivo complementation ability correlated well with the in vitro catalytic activities observed for these ribonuclease variants.

Crystallization of Minimal RNase E Fragments — In conjunction with the biochemical experiments described above, we attempted crystallization of the active RNase E fragments. The E. coli Rne 400 protein crystallized readily from 0.65 M sodium malonate, 20 mM MgCl₂, pH 5.0, and the crystals diffracted to ~6-Å resolution on a synchrotron beamline. E. coli Rng 400 failed to crystallize. The H. influen-
**RNase E Minimal Catalytic Domain**

enazee RNase G fragment crystallized readily from 3.5 m sodium acetate, pH 4.0, but the crystals diffracted poorly. The *H. influenzae* RNase E fragment crystallized under several different conditions; crystals grown from 1.8 M (NH₄)₂SO₄, 20 mM CoCl₂, and 100 mM MES, pH 6.5, diffract to ~3.4 Å (space group P6₁2₁2; a = 105.80 Å, c = 456.54 Å; native data have been collected to 3.4 Å resolution with *R*ₜₚ = 0.076). The *H. influenzae* RNase E fragment 1–399, from which the putatively extraneous amino-terminal residues were deleted, was also expressed and purified, and it crystallized with unit cell parameters and diffraction limits similar to those of the ~16 to 399 fragment.

**DISCUSSION**

In *E. coli*, RNase E is essential to cell viability (3, 38, 39) and is required for the processing or degradation of multiple RNAs. Homologs of RNase E have been conserved widely during bacterial evolution, and certain of these homologs also have been shown experimentally to have an important biological role in their native species (26, 27, 40) (also see review in Ref. 41). Earlier work has shown that the 498-residue amino-terminal segment of *E. coli* RNase E (i.e. N-Rne), which encompasses a fragment in which the amino acid sequence is highly conserved across all members of RNase E/G families, retains the structural features needed to enable the enzyme to cleave at specific RNA sites (17), confers a quasi-processive 3’ to 5’ scanning mode of enzymatic action (28, 42), and supports bacterial viability. However, an Rne protein fragment containing only the first 321 amino acid residues was inactive (17). The carboxyl-terminal (“scaffold”) region enhances the activity of the RNase E protein (29, 43) and is required for assembly of degradosomes containing polynucleotide phosphorylase, enolase, and the RhIB helicase (29), who found that a fusion protein consisting of 427 amino-terminal extension of RNase E participates in quaternary interaction and is required in its entirety for activity was provided by Ow et al. (29) who found that a fusion protein consisting of 427 amino-terminal residues plus 25 carboxyl-terminal residues, when overexpressed ~20-fold *in vivo*, could rescue an *rne* deletion. However, the cells were described as “viable but very defective in mRNA decay at 37 °C and nonviable at 44 °C,” and the enzymatic properties of the truncated protein were not examined (29).

We undertook to systematically define the minimal catalytic fragment necessary for the enzyme to function properly *in vitro* and *in vivo* and to elucidate the structural components required for these functions. Additionally, current models suggest that proper functioning of RNase E requires that the enzyme form a tetrameric quaternary structure, and we wished to learn the biochemical effects of removal of the Zn-link region recently found to be necessary for tetramer formation (36). Our results show that Rne protein fragments that contain ~400 amino acid residues of the ~1000-residue RNase E enzymes of *E. coli* and *H. influenzae* and lack the Zn-link retain the structural determinants required for site-specific endonuclease activity and the unique quasi-processive 3’ to 5’ scanning mode of action characteristic of RNase E *in vitro*.

Recently, Callaghan et al. (30) solved the crystal structure of the catalytic domain of *E. coli* RNase E by co-crystallizing the peptide containing amino acids 1–529 and RNA oligomeric substrate; they found that the 529-amino acid residue segment of the protein consists of two separate domains, a small one and a large one. The two domains of the 529-amino acid peptide analyzed by Callaghan et al. (30) are joined by a Zn-link, and their analysis of the crystal structure suggested that the Zn-link positions small domains of individual RNase E molecules to form a homotetrameric quaternary structure rather than link two dimers as proposed previously (36). Our computational analysis of the published crystal structure using a protein interaction server (Protein-Protein Interaction Server, version 1.5) found that the region likely to provide the most stable interface for interaction between the RNase E catalytic domains also spans the Zn-link and the small domain. Our biochemical evidence that a peptide virtually congruent with the large domain of Callaghan et al. (30) (i.e. the first 400 amino acid residues) is sufficient for RNase E catalytic activity, site-specific cleavage of substrates, and a 3’ to 5’ scanning mode of action is consistent with the observation that the catalytically functional minimal RNase E peptide region exists predominantly as a monomer under the conditions we employed for our analyses.

It has been shown that RNase E homologs, including *E. coli* RNase G (12) and *Streptomyces coelicolor* RNase ES (26), confer colony forming ability on an *rne* null mutant strain when overexpressed in *E. coli*, suggesting that RNase E-like proteins that differ significantly outside the conserved N-Rne fragment have similar biological abilities. Although in some cases, significantly greater induction of gene expression was required for functional complementation by the truncated peptides we studied, our results indicate that peptides that cleaved the RNA substrates used in these studies *in vitro* also had the ability, when expressed *in vivo*, to complement the *rne* null mutation. Conversely, truncated fragments of RNase G that showed no detectable enzymatic activity on a substrate containing specific RNase E cleavage sites failed to complement the *rne* null mutation at any level of induction tested. The RNase E/G fragments that we have characterized provide both positive and negative examples that establish a close correlation between *in vitro* endonuclease activity on an RNase E substrate and the capability to complement an *rne* null mutation *in vivo*.

The structural integrity of the minimal peptide fragments was inferred from the observed enzymatic and biological properties of the fragments and was further confirmed by their crystallization. Shorter, structurally stable fragments ~300 residues in length were isolated, but they did not retain enzymatic activ-

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*[^6]* *R*ₜₚ = ∑|[I]₁/₃ₖₙₜ – [I]ₕₖₙₜ|/|∑[I]ₕₖₙₜ|, where [I]ₕₖₙₜ = single value of measured intensity of hkl reflection, and [I]₁/₃ₖₙₜ = mean of all measured value intensities of hkl reflection.
ity. The requirement for a minimum of 395 amino acids for endonuclease activity, site-specific cleavage, and a quasi-processive mode of action of RNase E suggests that both the S1 domain near the amino-terminal end of the protein and the small arginine-rich region spanning amino acid residues 307 to 390 are essential to substrate binding and cleavage. However, the ~100 amino acids that follow this fragment, although highly conserved among RNase E/G homologs, are not essential. Amino-terminal (~400 residues) fragments of the RNase G proteins of *E. coli* and *H. influenzae* also displayed structural integrity, as demonstrated by crystallization of the fragment of the *H. influenzae* protein. However, they did not show endonuclease activity on the RNase E substrates used in these studies, and correspondingly, they did not complement an *rne* mutation in *vivo*.

Our results indicating that a peptide that lacks the Zn-link segment required for tetramer formation can nevertheless carry out the core catalytic functions of the enzyme argue strongly that tetramer formation is unnecessary for the enzyme to bind to the phosphate at the 5′ termini of RNA substrates (45) and then scan for cleavage sites from the 3′ end (28). The Zn-link region is also not necessary to confer colony-forming ability on an *rne* null mutant. We postulate that tetramer formation by RNase E instead may assist RNase E in maintaining RNA substrates in close proximity to the enzyme to facilitate efficient cleavage.

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