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# Identification of Amino Acid Residues in the Catalytic Domain of RNase E Essential for Survival of *Escherichia coli*: Functional Analysis of DNase I Subdomain

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ABSTRACT

RNase E is an essential *Escherichia coli* endoribonuclease that plays a major role in the decay and processing of a large fraction of RNAs in the cell. To better understand the molecular mechanisms of RNase E action, we performed a genetic screen for amino acid substitutions in the catalytic domain of the protein (N-Rne) that knock down the ability of RNase E to support survival of *E. coli*. Comparative phylogenetic analysis of RNase E homologs shows that wild-type residues at these mutated positions are nearly invariably conserved. Cells conditionally expressing these N-Rne mutants in the absence of wild-type RNase E show a decrease in copy number of plasmids regulated by the RNase E substrate RNA I, and accumulation of 5S ribosomal RNA, M1 RNA, and $tRNA^{Asn}$ precursors, as has been found in Rne-depleted cells, suggesting that the inability of these mutants to support cellular growth results from loss of ribonucleolytic activity. Purified mutant proteins containing an amino acid substitution in the DNase I subdomain, which is spatially distant from the catalytic site posited from crystallographic studies, showed defective binding to an RNase E substrate, p23 RNA, but still retained RNA cleavage activity—implicating a previously unidentified structural motif in the DNase I subdomain in the binding of RNase E to targeted RNA molecules, demonstrating the role of the DNase I domain in RNase E activity.

AMONG the many factors involved in the degradation and processing of RNA molecules in *Escherichia coli*, an endoribonuclease, RNase E, has been shown to play a major role in these processes. It is a multifunctional ribonuclease that degrades bulk RNA (Ono and Kuwano 1979), initiates the decay of a large fraction of mRNA (for recent reviews, see Coburn and Mackie 1999; Steege 2000) and regulatory RNAs (Masse *et al.* 2003; Morita *et al.* 2005) by cleaving them at highly specific sites, and assists in the maturation of a variety of catalytic RNAs, including 10Sa RNA (Lin-Chao *et al.* 1999), M1 RNA (Gurevitz *et al.* 1983), 5S rRNA (Ghora and Apirion 1978), and 16S rRNA (Li *et al.* 1999; Wachi *et al.* 1999).

The essential 118-kDa protein encoded by *rne* contains 1061 amino acids that can be partitioned into three functionally distinct domains (Casarégola *et al.* 1992). The catalytic function of RNase E resides in the N-terminal half of the protein (amino acid residues 1–498), which also contains cleavage site specificity (McDowall *et al.* 1995). Smaller RNase E derivatives that contain the first 395 amino acid residues show a weak cleavage activity *in vitro* and further truncation leads to loss of enzymatic activities (Caruthers *et al.* 2006). A recent study of the structure of RNase E further divides the catalytic domain into several subdomains: the RNase H, S1, 5′ sensor, DNase I, Zn, and small domains (Callaghan *et al.* 2005). The arginine-rich RNA-binding domain located between amino acids 580 and 700 is similar to one found in many RNA-binding proteins (Taraseviciene *et al.* 1995), and the C-terminal third of the RNase E protein serves as a scaffold for the formation of a multicomponent "degradosome" complex composed of the 3′ exonuclease polynucleotide phosphorylase (PNPase), the RNA helicase RhlB, and the glycolytic enzyme enolase (Carpousis *et al.* 1994; Miczak *et al.* 1996; Py *et al.* 1996; Vanzo *et al.* 1998; Liou *et al.* 2001; Leroy *et al.* 2002). For a recent review, see Carpousis (2007). RNase E has additionally been

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shown to be capable of interacting with poly(A) polymerase (Raynal and Carpousis 1999), ribosomal protein S1 (Kalapos *et al.* 1997; Feng *et al.* 2001), RNA-binding protein Hfq (Morita *et al.* 2005), and the protein inhibitors of RNase E activity, RraA and RraB (Lee *et al.* 2003; Gao *et al.* 2006). However, the N-terminal half (amino acid residues 1–498) is sufficient for cell survival (Kido *et al.* 1996; Ow *et al.* 2000).

Although significant progress has been made in determining the functional importance of RNase E in the degradation and processing of RNA transcripts (for review, see Coburn and Mackie 1999; Steege 2000) and the crystal structure of RNase E has been resolved (Callaghan *et al.* 2005), there is still limited understanding of the amino acid residues and structural motifs that mediate RNase E binding to and cleavage of specific *in vivo* RNA substrates, its 5′ → 3′ quasi-processive mode of enzyme action (Caruthers *et al.* 2006), and its 5′-end dependence (Mackie 1998). While studies of RNase E variants have revealed some of this information (Diwa *et al.* 2002; Briegel *et al.* 2006), an intensive and systematic search for RNase E loss-of-function mutants containing amino acid substitutions in the catalytic domain has not been done. To identify loss-of-function RNase E mutants, we developed a genetic system that allows the introduction of random mutations into the coding region of the catalytic domain, expression of the mutant RNase E proteins, and detection of mutant phenotypes in cells complemented in *trans* to allow bacterial cell growth. Using this approach, we identified residues in the catalytic domain important for ribonucleolytic activity. We report here the results of a systematic search for isolation and characterization of RNase E mutants showing a loss-of-function phenotype.

## MATERIALS AND METHODS

**Introduction of random mutations in the coding region of the catalytic domain of Rne:** To construct pNRNE4 plasmid (Tamura *et al.* 2006) containing random mutations in the coding region of N-Rne, gel-purified error-prone PCR products digested with *Not*I and *Xba*I were ligated into pNRNE4 plasmid that was digested with the same restriction enzymes. The DNA fragment encoding N-Rne was mutagenized by amplifying it using error-prone PCR as previously described (Kim *et al.* 2006). Primers used were Nrne 5′ (5′-GAATTGT GAGCGGATAAC-3′) and Nrne 3′ (5′-CTACCATCGGCGC TACGT-3′).

**Isolation and analysis of noncomplementing N-Rne mutants:** KSL2000 cells harboring pNRNE4-mut, which has random mutations in the coding region of the catalytic domain of RNase E, were individually tested on LB–agar medium containing 1–1000 μM IPTG to identify their ability to support the growth of KSL2000 cells expressing mutant N-Rne only. Three of the mutations isolated (I41N, A326T, and L385P) were subcloned into pLAC-RNE1-ΔH by ligating the *Not*I–*Pml*I fragment of pNRNE4 containing the mutations into the same sites in pLAC-RNE1-ΔH. Plasmid pLAC-RNE1-ΔH was constructed by ligating the *Hin*dIII–*Sph*I fragment of pFUS1500 (McDowall *et al.* 1995) containing the coding region for the C-terminal half of Rne into the *Hin*dIII–*Xba*I sites in pNRNE4.

**Protein work:** N-Rne or mutant N-Rne proteins were purified from KSL2000 cells harboring pNRNE4 or pNRNE4 containing mutations and Western blot analyses of Rne, N-Rne, and N-Rne-NC were carried out as described previously (Lee *et al.* 2002). Affinity purification of N-Rne protein typically yields >95% purity (supplemental Figure S2). To measure CD spectra of N-Rne and N-Rne-L385P proteins, purified proteins were stored in a buffer containing 20 mM Na $H_2PO_4$ (pH 7.5) and 200 mM NaCl at a concentration of 0.5 mg/ml. To prepare total proteins from KSL2000 + pACYC177 (no arabinose) or KSL2000 + pNRNE4 or pNRNE4-NC, cultures were grown to middle log phase in the presence of 0.1% arabinose, harvested, washed twice with plain Luria–Bertani (LB) medium, and reinoculated into LB medium containing no arabinose ($OD_{600} = 0.1$). They were further incubated for 150 min ($OD_{600} = 0.5$) at 37° and 250 rpm and harvested for total protein preparation.

***In vitro* cleavage of BR13:** Synthesis of 5′-end-labeled BR13 and universally labeled p23 RNA, gel mobility assay, cleavage assay, and Northern blot analysis were performed as described previously (Lee *et al.* 2003). The RNA bands in the gel were detected using a Packard Cyclone Phosphorimager and the intensity of each band was quantitated using OptiQuant.

## RESULTS

**A screening strategy to identify functional residues in the catalytic domain of RNase E:** Genetic analysis of RNase E has been hampered by the fact that it is an essential protein in *E. coli*. To circumvent this problem, we utilized an *E. coli* strain (KSL2000) in which a chromosomal deletion in *rne* is complemented by a plasmid-born *rne* gene under the control of an arabinose-inducible promoter (pBAD-RNE) (Lee *et al.* 2002). Addition of 0.1% arabinose to cultures of KSL2000 induces the synthesis of full-length RNase E at wild-type levels and consequently supports survival and growth of this *rne* deletion mutant; KSL2000 cells are unable to form colonies in the absence of arabinose (Tamura *et al.* 2006).

A compatible ampicillin-resistance ($Ap^r$) plasmid (pNRNE4) expressing the N-terminal 498 amino acids of RNase E with a hexahistidine tag at the C terminus (N-Rne) under the control of the isopropyl-thiogalactoside (IPTG)-inducible *lacUV5* promoter was introduced into KSL2000 (Figure 1A) and the resulting transformants were able to grow optimally in the presence of 10–100 μM IPTG (Figure 1B). Under these conditions, the steady-state level of N-Rne protein is about four times the normal level of full-length Rne, as determined by Western blot analysis using antibody against N-Rne, and is sufficient for optimal growth of the *rne* deletion mutant as previously reported (Lee *et al.* 2002; Lee and Cohen 2003). No full-length RNase E protein was detected in N-Rne-complemented bacteria (Figure 1C).

To identify functional residues in the catalytic domain of RNase E, the DNA segment encoding amino acids 1–498 of Rne was amplified using error-prone PCR, ligated into pNRNE4 by replacing the wild-type copy of *N-rne*, and introduced by transformation into KSL2000; transformants were individually tested for their ability

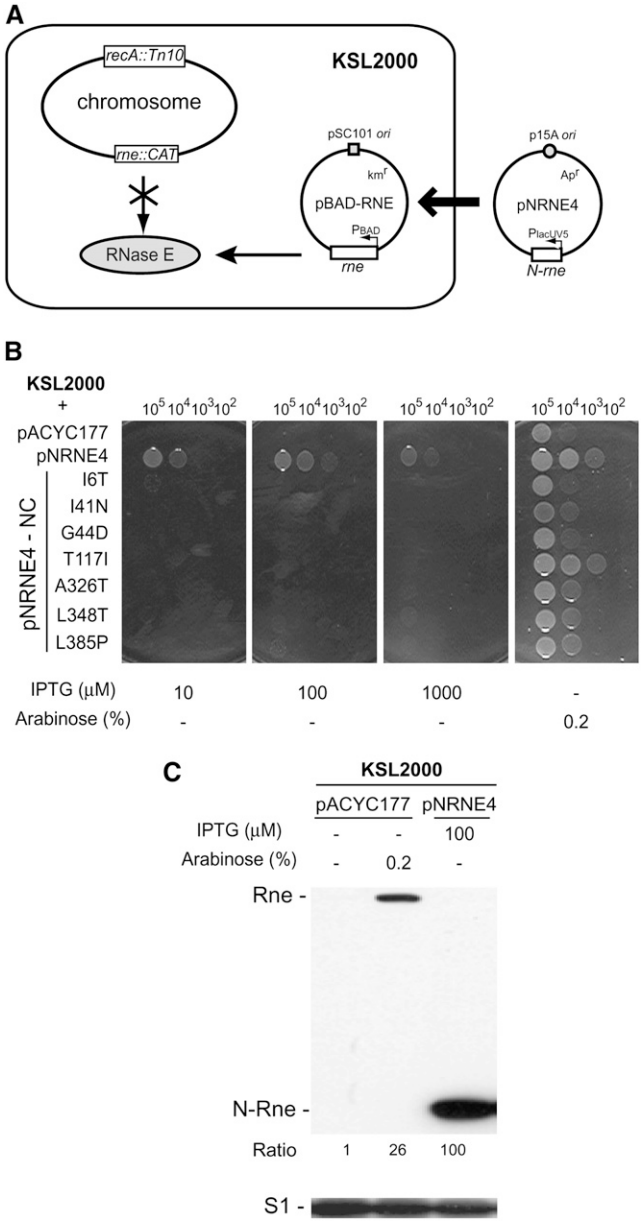


Figure 1.—Properties of the genetic system. (A) A genetic system to isolate N-Rne mutants. A compatible ampicillin resistance ($Ap^r$) plasmid (pNRNE4) expressing the N-terminal 498 amino acids of RNase E with a hexahistidine tag at the C terminus (N-Rne) harboring random amino acid substitutions under the control of the isopropyl-thiogalactoside (IPTG)-inducible lacUV5 promoter was introduced into KSL2000 in which a chromosomal deletion in *rne* was complemented by a plasmid-borne *rne* gene under the control of an arabinose-inducible BAD promoter (pBAD-RNE). (B) Growth characteristics of cells expressing noncomplementing N-Rne mutants. KSL2000 cells harboring pNRNE4 or pNRNE4-NC were individually tested on LB–agar medium containing 10–1000 μM IPTG for their ability to support the growth of KSL2000 cells. KSL2000 containing pACYC177 grew only when full-length RNase E was expressed from pBAD-RNE. (C) Expression profiles of Rne and N-Rne in KSL2000. The membrane probed with anti-Rne monoclonal antibody was stripped and subsequently reprobed with anti-S1 polyclonal antibody to provide an internal standard. The relative abundance of protein bands was quantitated using the Versa Doc imaging system and Quantity One.

to support the growth of KSL2000 cells on LB–agar medium containing 10–1000 μM IPTG. $MnCl_2$ (0.1 mM) was added to the PCR reaction to induce approximately one to three nucleotide substitutions per amplified copy, as has been previously determined by the random mutagenesis of a DNA fragment of similar size (~1.5 kbp) encoding 16S rRNA (Kim *et al.* 2006).

**Identification of functional residues in the catalytic domain of RNase E:** A total of 15,000 transformants harboring pNRNE4 containing random mutations in the coding region of N-Rne were screened for the loss of ability to support colony formation by KSL2000 cells in the presence of 10–1000 μM IPTG. Sixty-eight clones were obtained, and Western blot analysis using antibodies against RNase E showed that 12 of these expressed truncated proteins as a result of introduction of nonsense mutations (data not shown). Clones expressing truncated proteins were excluded from further analysis and the mutated residues of the rest of the clones were identified. As shown in Figure 2A, 18 clones contain a single-amino-acid substitution while the others contain two to three substitutions (not shown). The single-amino-acid substitutions cluster mainly in the DNase I, RNase H, and S1 subdomains and are positioned on the same surface of the protein that has been shown to bind and cleave RNA (Figure 2B) (Callaghan *et al.* 2005).

The degree of conservation of the wild-type amino acid residues that were substituted in the noncomplementing N-Rne (N-Rne-NC) mutants was analyzed by comparing the amino acid sequences of *E. coli* RNase E homologs found in other bacterial chromosomal DNA sequences in the NCBI database. The results show that the wild-type residues are nearly invariably conserved among RNase E homologs in phylogenetically diverse bacterial species (Table 1).

**Decay of RNA I by N-Rne-NC *in vivo*:** Seven of the noncomplementing mutants harboring a single-amino-acid substitution were further characterized to determine the basis of the inability of these mutants to complement a deficiency of wild-type N-Rne. KSL2000 cells expressing N-Rne-NC containing a single-amino-acid substitution in the RNase H (I6T), S1 (I41N, G44D, and T117I), or DNase I (A326T, I348T, and L385P) subdomain were conditionally expressed in the absence of full-length RNase E to determine the ribonucleolytic activity of the mutants in the cell. KSL2000 cells conditionally depleted for Rne by transferring bacteria to liquid media lacking arabinose underwent two to three cell divisions at a doubling rate similar to that observed for bacteria induced by 0.2% arabinose to express Rne at endogenous levels (data not shown). This result is consistent with the previous finding showing that *E. coli* cell division requires a cellular RNase E concentration at least 10–20% of normal (Jain *et al.* 2002).

Using this characteristic of KSL2000, we analyzed the steady-state level of a well-studied RNase E substrate in KSL2000 cells conditionally expressing N-Rne-NC in

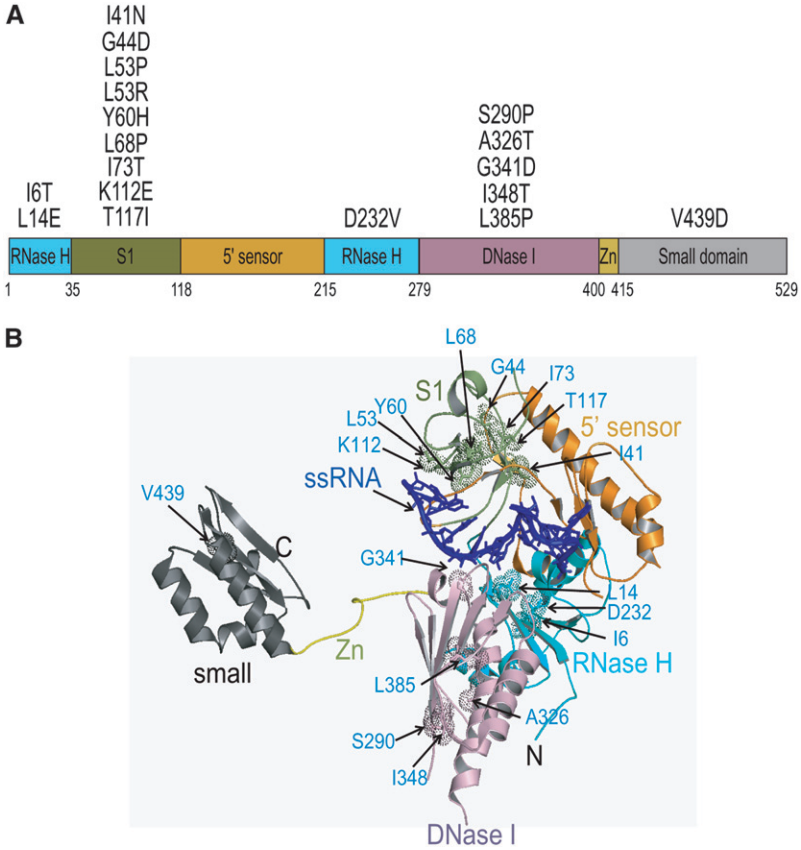


FIGURE 2.—Distribution of amino acid substitutions eliminating the ability of N-Rne to support the growth of *E. coli*. (A) The N-terminal domain (residues 1–529) of RNase E is divided into subdomains as indicated. Isolated single-amino-acid substitutions eliminating N-Rne's ability to support growth of *E. coli* are indicated above, respectively, the rectangle representing the N-terminal domain. (B) Isolated single-amino-acid substitutions are positioned in the crystal structure of the N-terminal domain of RNase E (CALLAGHAN *et al.* 2005).

the absence of the full-length RNase E to determine whether the inability of N-Rne mutants to support cell viability results from the loss of cellular ribonucleolytic activity. This RNase E substrate was RNA I, an antisense regulator of ColE1-type plasmid DNA replication; as previously shown RNA I abundance controls the copy number of the plasmid (LIN-CHAO *et al.* 1994) and this function has been used to assess the ribonucleolytic activity of RNase E *in vivo* (LEE and COHEN 2003; YEOM and LEE 2006). The induced expression of the N-Rne-NC mutants in the absence of wild-type RNase E in KSL2000 cells resulted in a decrease in copy number of the ColE1-type plasmid pNRNE4, which contains the noncomplementing mutations (pNRNE4-NC), by three to fourfold relative to that observed in KSL2000 cells expressing wild-type N-Rne (Figure 3A). Western blot analysis of N-Rne-NC proteins revealed that the amount of N-Rne-NC proteins in these cells was ~70–80% of wild-type N-Rne due to the decreased copy number of the plasmid that expresses N-Rne-NC (Figure 3B). These results show that N-Rne-NC mutants have lost the ability to cleave RNA I molecules and consequently have a lower copy number of the ColE1-type plasmid. Changes in copy number in cells expressing N-Rne-NC are likely to be amplified because pNRNE4 is a ColE1-type plasmid; however, changes in copy number do linearly reflect the extent of decrease in RNase E activity.

**Processing of essential noncoding RNAs in an *rne*-deleted strain expressing N-Rne-NC:** The initial discovery of RNase E was based upon its ability to process 9S rRNA in *E. coli* cells and the finding that a shift of *rne ts* bacteria to a nonpermissive temperature leads to the

**TABLE 1**

**Conservation of amino acid residues in RNase E-like proteins**

| Amino acid position | Amino acid | Conservation | |
|---|---|---|---|
| | | Identity | Similarity |
| 6 | Ile | 83/83 | 83/83 |
| 14 | Ile | 82/83 | 83/83 |
| 41 | Ile | 83/83 | 83/83 |
| 44 | Gly | 83/83 | 83/83 |
| 53 | Leu | 83/83 | 83/83 |
| 60 | Tyr | 71/83 | 83/83 |
| 68 | Leu | 83/83 | 83/83 |
| 73 | Ile | 81/83 | 83/83 |
| 112 | Lys | 83/83 | 83/83 |
| 117 | Thr | 83/83 | 83/83 |
| 232 | Asp | 83/83 | 83/83 |
| 290 | Ser | 81/83 | 81/83 |
| 326 | Ala | 83/83 | 83/83 |
| 341 | Gly | 83/83 | 83/83 |
| 348 | Ile | 83/83 | 83/83 |
| 385 | Leu | 83/83 | 83/83 |
| 439 | Val | 72/83 | 83/83 |

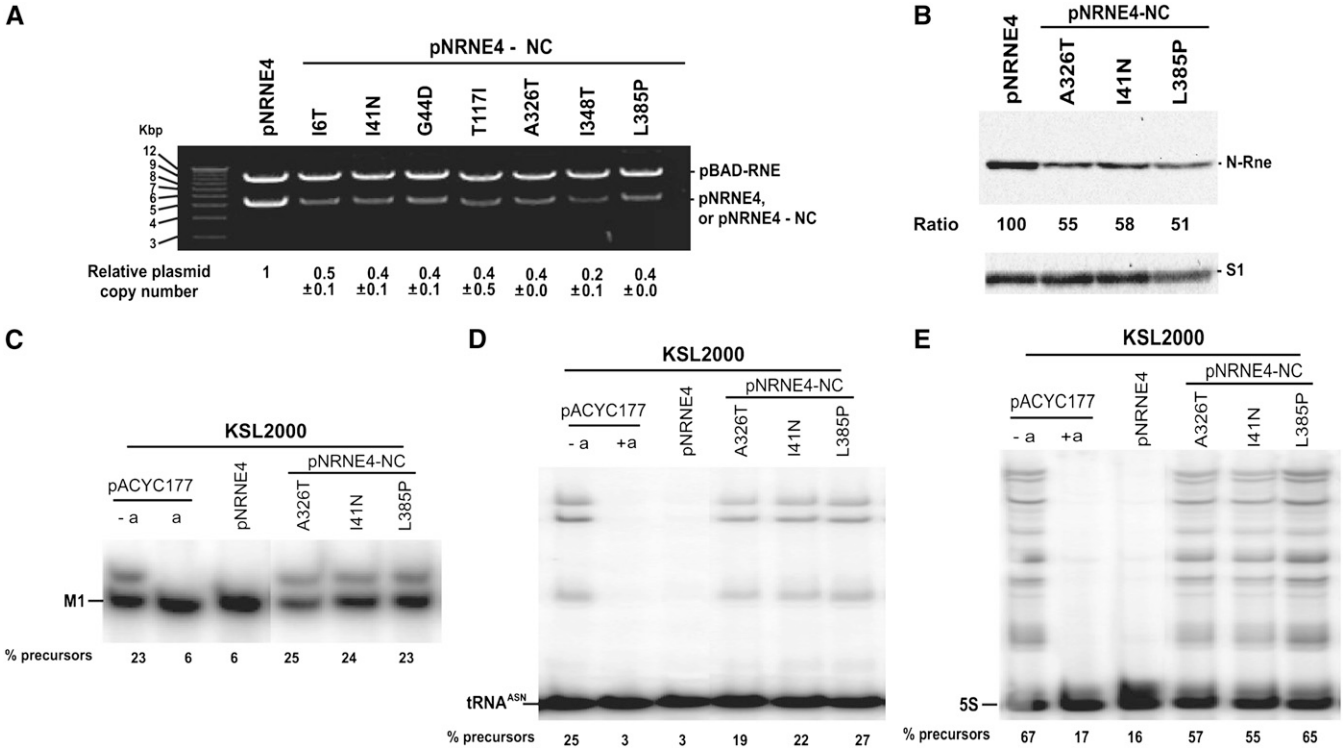


Figure 3.—Effect of N-Rne-NC on the stability of RNase E substrate RNAs *in vivo*. (A) Decay of RNA I. KSL2000 cells harboring pNRNE4 or pNRNE4-NC were depleted for Rne as described in materials and methods for plasmid preparation. Plasmids digested with *Hin*dIII restriction enzyme, which has a unique cleavage site in all plasmids tested here, were electrophoresed in a 0.9% agarose gel and stained with ethidium bromide. Plasmid copy numbers were calculated relative to a concurrently present pSC101 derivative (pBAD-RNE), the replication of which is independent of Rne, by measuring the molar ratio of the pBAD-RNE plasmid to ColE1-type plasmid (pNRNE4 or pNRNE-NC), and are shown at the bottom of the gel. (B) Expression profiles of N-Rne and N-Rne-NC in KSL2000 conditionally depleted for Rne. The same procedure described in the Figure 1C legend was used for Western blot analysis. Processing of pM1 (C), tRNA$^{Asn}$ (D), and 9S (E) is shown. Total RNA was separated in a 6% (pM1) or an 8% (tRNA$^{Asn}$ and 9S) PAGE containing 8 M urea. Separated RNA bands were transferred to a Nylon membrane and probed with $^{32}$P end-labeled oligos complementary to each RNA molecule. Percentages of precursors were calculated as previously described (Lee and Cohen 2003). KSL2000 cells expressing N-Rne-NC were grown for plasmid and total RNA preparation as described in materials and methods. This procedure was applied to remove the full-length RNase E expressed from pBAD-RNE and to measure the ribonucleolytic activity of N-Rne mutants in the cell.

*in vivo* accumulation of precursors of 5S rRNA (Ghora and Apirion 1978), pM1 RNA (Reed *et al.* 1982), and tRNA$^{Asn}$ (Li and Deutscher 2002; Ow and Kushner 2002). To test the ability of N-Rne-NC mutants to process precursors of these essential noncoding RNA transcripts, we measured the steady-state transcript levels in cells conditionally expressing N-Rne-NC mutants containing a single-amino-acid substitution in the S1 subdomain (I41N), which previously has been shown to be implicated in the binding of RNase E to substrate RNA (Schubert *et al.* 2004; Callaghan *et al.* 2005), and in the DNase I subdomain (A326T and L385P), which is spatially distant from the catalytic and RNA-binding site of the protein (Callaghan *et al.* 2005). We found that Rne-depleted cells expressing these N-Rne-NC mutants were deficient in the processing of precursors of all of these RNAs (Figure 3, C–E). The precursor bands accumulating in *rne*-deleted cells expressing the noncomplementing N-Rne mutants were identical in size to, and similar in quantity to, the species accumulating in *rne*-deleted cells in which synthesis of RNase E from the pBAD-RNE plasmid (*i.e.*, the KSL2000 strain) was turned off by shift to media lacking arabinose (Figure 3, C–E, lane 1). In contrast, the RNAs were processed normally in KSL2000 cells expressing wild-type N-Rne (Figure 3, C–E, lane 3). These results suggest that these N-Rne-NC mutants have little to no ribonucleolytic activity *in vivo*.

**Effects of noncomplementing single-amino-acid substitutions on the ribonucleolytic activity of full-length RNase E:** To confirm that the loss of ribonucleolytic activity by substitution of a single amino acid in these mutant proteins is not a property of only the truncated N-Rne protein, three of the mutations (I41N, A326T, and L385P) were subcloned into a plasmid expressing full-length RNase E under the control of the IPTG-inducible *lacUV5* promoter (pLAC-RNE1-ΔH) and tested for their ability to support the growth of KSL2000 cells in the presence of 1–1000 μM IPTG. While KSL2000 cells expressing wild-type RNase E from pLAC-RNE1-ΔH

grew normally in the presence of 1–10 μM IPTG, none of the RNase E mutants containing a noncomplementing mutation supported the growth of KSL2000 at IPTG concentrations of 1–1000 μM, indicating that the effects of these amino acid substitutions are not specific to N-Rne (data not shown).

***In vitro* cleavage activity of N-Rne-NC:** To test whether the observed evidence of decreased ribonucleolytic activity of N-Rne-NC mutants *in vivo* resulted from defective ribonucleolytic activity of the mutated N-Rne-NC proteins, we measured the *in vitro* cleavage rates of wild-type and N-Rne-NC proteins on BR13, an oligoribonucleotide that contains the RNase E-cleaved sequence of RNA I. Affinity-purified wild-type or mutant N-Rne proteins containing a single-amino-acid substitution (I41N, A326T, and L385P) were incubated with 5′-end-labeled BR13. None of the three N-Rne-NC proteins tested detectably cleaved BR13, whereas this oligoribonucleotide was cleaved efficiently by the wild-type N-Rne protein (Figure 4A).

**Characterization of N-Rne-NC proteins bearing mutations in the region of DNase I subdomain spatially distant from the catalytic site:** We were particularly interested in learning the basis for the observed loss of ribonucleolytic function for mutants containing single-amino-acid substitutions within the DNase I subdomain, which is not in close proximity to the site implicated by crystallographic analysis of the N-Rne · BR13 complex (Figure 2B) in the binding and cleavage of BR13 (Callaghan *et al.* 2005). Although the functional role of this region has not been previously identified, mutations in the region could in principle interfere with the ribonucleolytic activity of RNase E by either reducing binding to the RNA substrate or inhibiting the catalytic activity of the enzyme. To further understand the basis for the loss of ribonucleolytic activity of these mutant proteins, we tested the ability of N-Rne-NC proteins mutated in the DNase I subdomain (A326T and L385P) to bind to p23 RNA, which is a truncated pM1 RNA that is processed by RNase E to a product termed 23 RNA (Kim *et al.* 1996). Gel shift assays indicated the inability of these N-Rne-NC mutants to bind to p23 RNA (Figure 4B). We detected the major band (band b in Figure 4B) that did not interact with wild-type protein and consequently did not shift. We think that it is probably p23 RNA molecules with a different structure that does not allow wild-type N-Rne to bind. Even though the *in vitro* synthesized p23 RNA was denatured at 75° and renatured by slowly cooling down to room temperature, two separate RNA bands (bands a and b) were formed in the gel. It is possible that the complex formed between wild-type protein and p23 RNA present in band a is more stable than the one formed between the protein and p23 RNA present in band b under the conditions used for the gel mobility shift assay. A similar phenomenon has been observed when an untruncated version of p23 RNA (pM1) was used for the gel mobility shift assay (Lee *et al.* 2003).

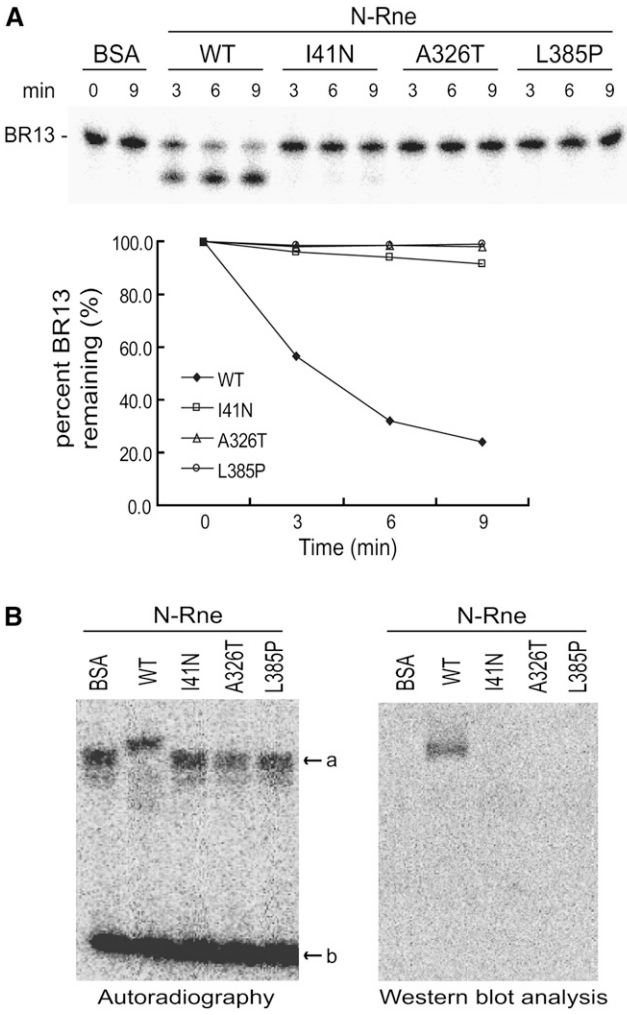


Figure 4.—(A) Cleavage of BR13 by N-Rne and N-Rne-NC *in vitro*. One-half picomole of 5′-end-labeled BR13 was incubated with 50 ng of purified N-Rne or N-Rne-NC in cleavage buffer at 37°. Each sample was removed at each time point indicated and mixed with an equal volume of loading buffer. Samples were denatured at 75° for 5 min and loaded onto 15% polyacrylamide gels containing 8 M urea. The radioactivity in each band was quantitated using phosphorimager and OptiQuant. (B) RNA-binding activity of N-Rne-NC. One-half picomole of internally labeled p23 RNA was incubated at room temperature for 10 min with 50 ng of proteins indicated in 20 μl of 1× cleavage buffer. After detecting RNA bands using phosphorimager, proteins in the gel was transferred to a nitrocellulose membrane and probed with monoclonal antibodies to Rne.

In contrast to wild-type N-Rne, the N-Rne-A326T and N-Rne-L385P mutants in the DNase I subdomain as well as the N-Rne-I41N mutant in the S1 subdomain all showed no detectable binding to p23 RNA under the same conditions, suggesting that the defective ribonucleolytic activity of N-Rne-A326T and N-Rne-L385P results from reduction in the substrate-binding ability of the enzyme. When higher concentrations of proteins were

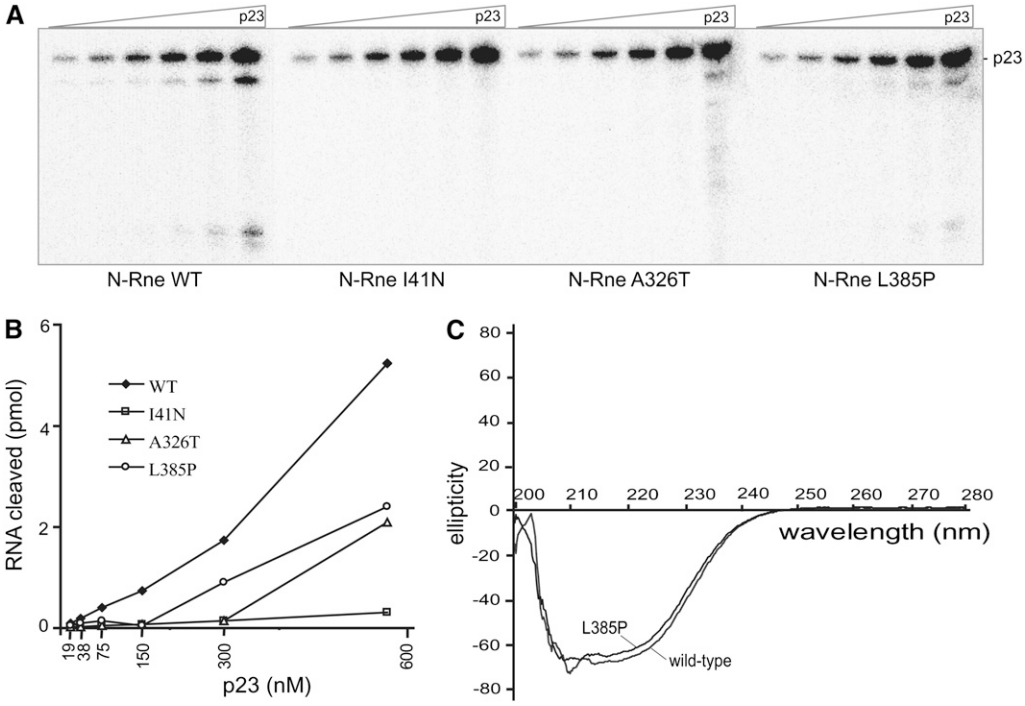


FIGURE 5.—Cleavage activity of N-Rne-NC. (A) *In vitro* cleavage of p23 RNA by N-Rne-NC. Each protein (wild type, I41N, A326T, or L385P) at the concentration of 20 nM was incubated with various concentrations of p23 (18.8–600 nM) for 15 min at 37° and analyzed in an 8% PAGE containing 8 M urea. (B) Quantitation of cleavage activity of N-Rne-NC. The radioactivity in each band was quantitated using phosphorimager and OptiQuant and plotted. (C) Detection of misfolding of N-Rne-L385P. Purified proteins of N-Rne and N-Rne-L385P were used to measure the CD spectrum.

used for the gel mobility shift assay, p23 RNA incubated with wild-type N-Rne was cleaved and consequently the uncleaved RNA bands (bands a and b in Figure 4B, lane 2) were converted to new bands below band a (lane 2 in supplemental Figure S1). However, including higher levels of mutant protein N-Rne-L385P did not result in a shift of any bands (lane 3 in supplemental Figure S1).

Consistent with this observation, at the highest substrate concentrations tested, cleavage products were observed for the N-Rne-A326T and N-Rne-L385P proteins, although the yield was much less than that for the wild-type enzyme (Figure 5, A and B). However, N-Rne-I41N, which failed to show any binding activity (Figure 4), showed no cleavage activity at any substrate concentration tested. As proline substitutions commonly lead to disruption of protein structure, we compared the structure of N-Rne-L385P protein with wild-type N-Rne, using circular dichroism (CD) to learn whether the mutation that abolishes the binding and enzymatic activity of the N-Rne-L385P protein leads to misfolding of the protein. As shown in Figure 5C, the CD spectrum of N-Rne-L385P protein was nearly identical to that of wild-type N-Rne, indicating that there is no significant collapse or misfolding of the mutant protein.

## DISCUSSION

The isolated single-amino-acid substitutions eliminating the ability of RNase E to support survival of *E. coli* cells are clustered in the S1, DNase I, and RNase H subdomains and positioned on the same face of the protein as the RNA-binding and cleavage sites. However, 11 functionally important residues identified in a recently resolved crystal structure of N-terminal RNase E complexed with a short (10–15 nt) RNA oligonucleotide having 2′-*O*-methyl modifications (CALLAGHAN *et al.* 2005) did not overlap with the noncomplementing residues isolated in this study except for the lysine residue at position 112. The functionally important residues identified by CALLAGHAN *et al.* (2005) are all implicated in engagement of the terminal phosphate (G124, V128, R169, T170, and R373), forming a hydrophobic pocket that binds the nucleotide adjacent to the cleavage site (F57, F67, and K112), nucleophilic attack of the scissile phosphate (D303, N305, and D346), and contact with the exocyclic oxygen of the base at position −1 with regard to the scissile phosphate (K106). As selective replacement by CALLAGHAN *et al.* (2005) of functionally important RNase E residues with other amino acids based on the crystal structure resulted in the loss of RNA-binding ability, RNA cleavage activity, or both *in vitro*, it was surprising to find only one overlapping position (K112E) among mutations that prevented RNase E from supporting cell viability. One of several possibilities may account for this absence of overlap. Although we screened 15,000 clones to isolate 18 noncomplementing mutants bearing single-amino-acid substitutions, we observed a low frequency of mutation redundancy, implying that the library of possible mutations was not fully saturated. Additionally, the amino acids chosen for mutagenesis based on the crystal structure were all implicated in contacts with small oligonucleotides, which are unlikely to be major RNase E substrates *in vivo* and, therefore, might not represent all functional residues of RNase E that interact with *in vivo* RNA substrates, which are much longer and more

complex than small oligonucleotides. It is also possible that some of the residues found in our mutants are not directly involved in binding or cleaving RNA, but rather in forming structural elements to maintain the active form of the enzyme. One such example is the mutation L68P recovered in our screen that had been previously identified in two conditional mutants (G66S and L68F) that lead to a lethal phenotype at elevated temperatures; these mutations were inferred to globally destabilize the folded structure of the RNase E S1 domain (Schubert *et al.* 2004). A final possibility is that the residues we identified mediate enzyme functions that cannot be inferred from the crystal structure. For example, it has been proposed that multimeric forms of RNase E can be catalytically activated by the allosteric effector 5′ monophosphate present in target RNAs, which induces significant structural changes in the protein that both enhance catalytic activity and constrain substrate binding (Jiang and Belasco 2004), and the enzyme may require such augmented binding and catalytic activity to cleave some substrates *in vivo* that are long structured RNAs.

The wild-type amino acid residues at the mutated positions we identified, which are mainly clustered in the S1, DNase I, and RNase H subdomains, are nearly invariably conserved in RNase E homologs, consistent with our finding that they are essential for cell survival. However, notwithstanding the essentiality of normal residues at these positions, two of the three the mutant proteins we tested bind to RNA (albeit poorly) and show *in vitro* ribonucleolytic activity at high substrate concentrations.

The ability of RNase E-like enzymes to cleave AU-rich single-stranded regions of numerous target RNA molecules implies that these proteins have a conserved structural motif for selecting cleavage sites present in structurally complex RNA substrates. As already noted, the only residue so far identified on the basis of the crystal structure for sequence recognition is K106, and it has been inferred that the preference of RNase E for AU-rich substrates results mainly through the recognition of the RNA conformation (Callaghan *et al.* 2005). However, it is known that the enzyme does not simply cleave AU-rich single-stranded regions of RNA molecules but rather cleaves RNA sequences with high specificity (Lin-Chao *et al.* 1994; McDowall *et al.* 1995; Kaberdin 2003). Moreover, *in vivo* RNase E substrates have cleavage sites that commonly are preceded or followed by a stem-loop structure that seems to modulate the degradation rate of the RNA transcripts (Melefors and Von Gabain 1988; Lin-Chao and Cohen 1991; Cormack and Mackie 1992; Mackie and Genereaux 1993; Diwa *et al.* 2000). Therefore, it is likely that the RNase E protein has additional RNA-binding motifs to select AU-rich single-stranded regions in highly structured RNA substrates. Our finding that mutant proteins bearing amino acid substitutions A326T and L385P fail to bind RNA but still retain catalytic activity suggests that the region of the DNase I subdomain that contains these amino acid substitutions and that is spatially distant from the catalytic site of RNase E may contain additional RNA-binding sequences or may modulate the binding of other enzyme regions to substrates. It also remains possible that misfolding of mutant proteins to a degree that was not detected by CD analysis is the basis for the phenotype. However, any such misfolding would likely be limited and localized, as some RNA cleavage activity is retained by the mutated protein. Rather, it is tempting to speculate that wild-type residues at these mutated positions in the DNase I subdomain may constitute a highly conserved structural motif that aids RNase E-like enzymes in the selection of specific cleavage sites in AU-rich, single-stranded regions. This motif may facilitate enzymatic attack on such regions by overcoming impediments imposed by *cis*- and/or *trans*-acting elements such as high-order RNA structures and RNA-binding proteins present in the vicinity of the cleavage sites. It is unlikely that all amino acid substitutions identified in the DNase I subdomain (Figure 2A) are implicated in forming the additional RNA-binding motif since the region proximal to the catalytic site of RNase E that includes D303, N305, and D346 has been proposed to interact with the hydrated magnesium ion, the activated water molecule that attacks the scissile phosphate of the RNA (Callaghan *et al.* 2005).

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## LITERATURE CITED

Briegel, K. J., A. Baker and C. Jain, 2006 Identification and analysis of *Escherichia coli* ribonuclease E dominant-negative mutants. Genetics **172:** 7–15.

Callaghan, A. J., M. J. Marcaida, J. A. Stead, K. J. McDowall, W. G. Scott *et al.*, 2005 Structure of *Escherichia coli* RNase E catalytic domain and implications for RNA turnover. Nature **437:** 1187–1191.

Carpousis, A. J., 2007 The RNA degradosome of *Escherichia coli*: an mRNA-degrading machine assembled on RNase E. Annu. Rev. Microbiol. **61:** 71–87.

Carpousis, A. J., G. V. Houwe, C. Ehretsmann and H. M. Krisch, 1994 Copurification of *E. coli* RNase E and PNPase: evidence for a specific association between two enzymes important in RNA processing and degradation. Cell **76:** 889–900.

Casarégola, S., A. Jacq, D. Laoudj, G. McGurk, S. Margarson *et al.*, 1992 Cloning and analysis of the entire *Escherichia coli ams* gene: *ams* is identical to hmp1 and encodes a 114 kDa protein that migrates as a 180 kDa protein. J. Mol. Biol. **228:** 30–40.

Caruthers, J. M., Y. Feng, D. B. McKay and S. N. Cohen, 2006 Retention of core catalytic functions by a conserved minimal ribonuclease E peptide that lacks the domain required for tetramer formation. J. Biol. Chem. **281:** 27046–27051.

Coburn, G. A., and G. A. Mackie, 1999 Degradation of mRNA in *Escherichia coli*: an old problem with some new twists. Prog. Nucleic Acid Res. Mol. Biol. **62:** 55–108.

Cormack, R. S., and G. A. Mackie, 1992 Structural requirements for the processing of *Escherichia coli* 5 S ribosomal RNA by RNase E *in vitro*. J. Mol. Biol. **228:** 1078–1090.

Diwa, A., A. L. Bricker, C. Jain and J. G. Belasco, 2000 An evolutionarily conserved RNA stem-loop functions as a sensor that di-

rects feedback regulation of RNase E gene expression. Genes Dev. **14:** 1249–1260.

Diwa, A. A., X. Jiang, M. Schapira and J. G. Belasco, 2002 Two distinct regions on the surface of an RNA-binding domain are crucial for RNase E function. Mol. Microbiol. **46:** 959–969.

Feng, Y., H. Huang, J. Liao and S. N. Cohen, 2001 *Escherichia coli* poly(A)-binding proteins that interact with components of degradosomes or impede RNA decay mediated by polynucleotide phosphorylase and RNase E. J. Biol. Chem. **276:** 31651–31656.

Gao, J., K. Lee, M. Zhao, J. Qiu, X. Zhan *et al.*, 2006 Differential modulation of *E. coli* mRNA abundance by inhibitory proteins that alter the composition of the degradosome. Mol. Microbiol. **61:** 394–406.

Ghora, B. K., and D. Apirion, 1978 Structural analysis and *in vitro* processing to p5 rRNA of a 9S RNA molecule isolated from an *rne* mutant of *E. coli*. Cell **15:** 1055–1066.

Gurevitz, M., S. K. Jain and D. Apirion, 1983 Identification of a precursor molecule for the RNA moiety of the processing enzyme RNase P. Proc. Natl. Acad. Sci. USA **80:** 4450–4454.

Jain, C., A. Deana and J. G. Belasco, 2002 Consequences of RNase E scarcity in *Escherichia coli*. Mol. Microbiol. **43:** 1053–1064.

Jiang, X., and J. G. Belasco, 2004 Catalytic activation of multimeric RNase E and RNase G by 5′-monophosphorylated RNA. Proc. Natl. Acad. Sci. USA **101:** 9211–9216.

Kaberdin, V. R., 2003 Probing the substrate specificity of *Escherichia coli* RNase E using a novel oligonucleotide-based assay. Nucleic Acids Res. **31:** 4710–4716.

Kalapos, M. P., H. Paulusb and N. Sarkara, 1997 Identification of ribosomal protein S1 as a poly(A) binding protein in *Escherichia coli*. Biochimie **79:** 493–502.

Kido, M., K. Yamanaka, T. Mitani, H. Niki, T. Ogura *et al.*, 1996 RNase E polypeptides lacking a carboxyl-terminal half suppress a *mukB* mutation in *Escherichia coli*. J. Bacteriol. **178:** 3917–3925.

Kim, J. M., H. Go, W. S. Song, S. M. Ryou and K. Lee, 2006 Functional analysis of the residue 789 in *Escherichia coli* 16S rRNA and development of a method to select second-site revertants. Korean J. Microbiol. **42:** 156–159.

Kim, S., H. Kim, I. Park and Y. Lee, 1996 Mutational analysis of RNA structures and sequences postulated to affect 3′ processing of M1 RNA, the RNA component of *Escherichia coli* RNase P. J. Biol. Chem. **271:** 19330–19337.

Lee, K., and S. N. Cohen, 2003 A *Streptomyces coelicolor* functional orthologue of *Escherichia coli* RNase E shows shuffling of catalytic and PNPase-binding domains. Mol. Microbiol. **48:** 349–360.

Lee, K., J. A. Bernstein and S. N. Cohen, 2002 RNase G complementation of rne null mutation identifies functional interrelationships with RNase E in *Escherichia coli*. Mol. Microbiol. **43:** 1445–1456.

Lee, K., X. Zhan, J. Gao, J. Qiu, Y. Feng *et al.*, 2003 RraA: a protein inhibitor of RNase E activity that globally modulates RNA abundance in *E. coli*. Cell **114:** 623–634.

Leroy, A., N. F. Vanzo, S. Sousa, M. Dreyfus and A. J. Carpousis, 2002 Function in *Escherichia coli* of the non-catalytic part of RNase E: role in the degradation of ribosome-free mRNA. Mol. Microbiol. **45:** 1231–1243.

Li, Z., and M. P. Deutscher, 2002 RNase E plays an essential role in the maturation of *Escherichia coli* tRNA precursors. RNA **8:** 97–109.

Li, Z., S. Pandit and M. P. Deutscher, 1999 RNase G (CafA protein) and RNase E are both required for the 5′ maturation of 16S ribosomal RNA. EMBO J. **18:** 2878–2885.

Lin-Chao, S., and S. N. Cohen, 1991 The rate of processing and degradation of antisense RNAI regulates the replication of ColE1-type plasmids *in vivo*. Cell **65:** 1233–1242.

Lin-Chao, S., T. T. Wong, K. J. McDowall and S. N. Cohen, 1994 Effects of nucleotide sequence on the specificity of *rne*-dependent and RNase E-mediated cleavages of RNA I encoded by the pBR322 plasmid. J. Biol. Chem. **269:** 10797–10803.

Lin-Chao, S., C. L. Wei and Y. T. Lin, 1999 RNase E is required for the maturation of *ssrA* RNA and normal *ssrA* RNA peptide-tagging activity. Proc. Natl. Acad. Sci. USA **96:** 12406–12411.

Liou, G.-G., W.-N. Jane, S. N. Cohen, N.-S. Lin and S. Lin-Chao, 2001 RNA degradosomes exist *in vivo* in *Escherichia coli* as multicomponent complexes associated with the cytoplasmic membrane via the N-terminal region of ribonuclease E. Proc. Natl. Acad. Sci. USA **98:** 63–68.

Mackie, G. A., 1998 Ribonuclease E is a 5′-end-dependent endonuclease. Nature **395:** 720–723.

Mackie, G. A., and J. L. Genereaux, 1993 The role of RNA structure in determining RNase E-dependent cleavage sites in the mRNA for ribosomal protein S20 *in vitro*. J. Mol. Biol. **234:** 998–1012.

Masse, E., F. E. Escorcia and S. Gottesman, 2003 Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*. Genes Dev. **17:** 2374–2383.

McDowall, K. J., V. R. Kaberdin, S. W. Wu, S. N. Cohen and S. Lin-Chao, 1995 Site-specific RNase E cleavage of oligonucleotides and inhibition by stem-loops. Nature **374:** 287–290.

Melefors, O., and A. von Gabain, 1988 Site-specific endonucleolytic cleavages and the regulation of stability of *E. coli ompA* mRNA. Cell **52:** 893–901.

Miczak, A., V. R. Kaberdin, C.-L. Wei and S. Lin-Chao, 1996 Proteins associated with RNase E in a multicomponent ribonucleolytic complex. Proc. Natl. Acad. Sci. USA **93:** 3865–3869.

Morita, T., K. Maki and H. Aiba, 2005 RNase E-based ribonucleoprotein complexes: mechanical basis of mRNA destabilization mediated by bacterial noncoding RNAs. Genes Dev. **19:** 2176–2186.

Ono, M., and M. Kuwano, 1979 A conditional lethal mutation in *Escherichia coli* strain with a longer chemical lifetime of messenger RNA. J. Mol. Biol. **129:** 343–357.

Ow, M. C., and S. R. Kushner, 2002 Initiation of tRNA maturation by RNase E is essential for cell viability in *E. coli*. Genes Dev. **16:** 1102–1115.

Ow, M. C., Q. Liu and S. R. Kushner, 2000 Analysis of mRNA decay and rRNA processing in *Escherichia coli* in the absence of RNase E-based degradosome assembly. Mol. Microbiol. **38:** 854–866.

Py, B., C. F. Higgins, H. M. Krisch and A. J. Carpousis, 1996 A DEADbox RNA helicase in the *Escherichia coli* RNA degradosome. Nature **381:** 169–172.

Raynal, L. C., and A. J. Carpousis, 1999 Poly(A) polymerase I of *Escherichia coli*: characterization of the catalytic domain, an RNA binding site and regions for the interaction with proteins involved in mRNA degradation. Mol. Microbiol. **32:** 765–775.

Reed, R. E., M. F. Baer, C. Guerrier-Takada, H. Donis-Keller and S. Altman, 1982 Nucleotide sequence of the gene encoding the RNA subunit (M1 RNA) of ribonuclease P from *Escherichia coli*. Cell **30:** 627–636.

Schubert, M., R. E. Edge, P. Lario, M. A. Cook, N. C. Strynadka *et al.*, 2004 Structural characterization of the RNase E S1 domain and identification of its oligonucleotide-binding and dimerization interfaces. J. Mol. Biol. **30:** 37–54.

Steege, D. A., 2000 Emerging features of mRNA decay in bacteria. RNA **6:** 1079–1090.

Tamura, M., K. Lee, C. A. Miller, C. J. Moore, Y. Shirako *et al.*, 2006 RNase E maintenance of proper FtsZ/FtsA ratio required for nonfilamentous growth of *Escherichia coli* cells but not for colony-forming ability. J. Bacteriol. **188:** 5145–5152.

Taraseviciene, L., G. R. Bjork and B. E. Uhlin, 1995 Evidence for an RNA binding region in the *Escherichia coli* processing endoribonuclease RNase E. J. Biol. Chem. **270:** 26391–26398.

Vanzo, N. F., Y. S. Li, B. Py, E. Blum, C. F. Higgins *et al.*, 1998 Ribonuclease E organizes the protein interactions in the *Escherichia coli* RNA degradosome. Genes Dev. **12:** 2770–2781.

Wachi, M., G. Umitsuki, M. Shimizu, A. Takada and K. Nagai, 1999 *Escherichia coli cafA* gene encodes a novel RNase, designated as RNase G, involved in processing of the 5′ end of 16S rRNA. Biochem. Biophys. Res. Commun. **259:** 483–488.

Yeom, J.-H., and K. Lee, 2006 RraA rescues *Escherichia coli* cells overproducing RNase E from growth arrest by modulating the ribonucleolytic activity. Biochem. Biophys. Res. Commun. **345:** 1372–1376.

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