Identification and Analysis of *Escherichia coli* Ribonuclease E Dominant-Negative Mutants

Karoline J. Briegel, Asmaa Baker and Chaitanya Jain¹

Department of Biochemistry and Molecular Biology, University of Miami Miller School of Medicine, Miami, Florida 33136

Manuscript received July 21, 2005

Accepted for publication September 27, 2005

ABSTRACT

The *Escherichia coli* (*E. coli*) ribonuclease E protein (RNase E) is implicated in the degradation and processing of a large fraction of RNAs in the cell. To understand RNase E function in greater detail, we developed an efficient selection method for identifying nonfunctional RNase E mutants. A subset of the mutants was found to display a dominant-negative phenotype, interfering with wild-type RNase E function. Unexpectedly, each of these mutants contained a large truncation within the carboxy terminus of RNase E. In contrast, no point mutants that conferred a dominant-negative phenotype were found. We show that a representative dominant-negative mutant can form mixed multimers with RNase E and propose a model to explain how these mutants can block wild-type RNase E function *in vivo*.

THE degradation of mRNAs in bacterial cells occurs rapidly, and mRNA half-lives of a few minutes or less are typical (COBURN and MACKIE 1999; STEEGE 2000). This instability has important consequences for gene expression and is believed to be important for cellular adaptation to changing environments. In *E. coli*, a number of potential ribonucleases that can participate in mRNA degradation and processing have been identified (DEUTSCHER and LI 2001; CONDON and PUTZER 2002). Among these factors, RNase E has been shown to have a major role in initiating the degradation of a large number of transcripts (COBURN and MACKIE 1999). In addition, RNase E plays important roles in the degradation of small regulatory RNAs and the maturation of transfer and ribosomal RNAs from their respective precursors (LI *et al.* 1999; LI and DEUTSCHER 2002; OW and KUSHNER 2002; MASSE *et al.* 2003).

RNase E is an endonuclease that cleaves mRNAs primarily within A–U rich, unstructured regions without the requirement for a stringent recognition sequence (LIN-CHAO *et al.* 1994; McDOWALL *et al.* 1994). Its N-terminal domain contains a region of homology common to ribosomal S1 protein and to many other ribonucleases (S1 domain), as well as the amino acids necessary for RNA cleavage (McDOWALL and COHEN 1996; BYCROFT *et al.* 1997; SCHUBERT *et al.* 2004). A central region of the protein contains an arginine-rich region, similar to one found in many RNA-binding proteins (TARASEVICIENE *et al.* 1995). The C-terminal region participates in interactions with a number of other *E. coli* proteins including the exonuclease polynucleotide phosphorylase, RhlB

helicase, and enolase, a glycolytic enzyme, to form a complex denoted the “degradosome” (CARPOUSIS *et al.* 1994; MICZAK *et al.* 1996; PY *et al.* 1996).

While the importance of RNase E in mRNA degradation is well appreciated, many details pertaining to RNase E function are still not well understood. For example, neither the specific amino acid residues that contribute to RNA cleavage nor those that discriminate substrates based on the RNA 5′-end have been identified. (LIN-CHAO and COHEN 1991; BOUVET and BELASCO 1992; MACKIE 1998). RNase E contains an arginine-rich, RNA-binding central domain, but its role in substrate recognition is unclear since RNase E variants lacking this domain remain capable of RNA cleavage (McDOWALL and COHEN 1996). In addition, structural information on RNase E is presently limited to a 91 amino-acid region that encompasses the S1 domain (SCHUBERT *et al.* 2004). Therefore, it is not yet feasible to accurately model RNA cleavage by RNase E.

Some of these details could be clarified by studying defective RNase E variants. One recent study focused on the role of positively charged lysine and arginine as well as surface aromatic residues present within the S1 domain of RNase E (DIWA *et al.* 2002). Two other studies have investigated the effects of creating deletions within the C-terminal region of RNase E (OW *et al.* 2000; LEROY *et al.* 2002). However, a systematic search for RNase E mutants remains to be undertaken. To provide a rapid means of identifying useful RNase E mutants, we have devised an efficient selection process, which is based on the ability of functionally defective but not functionally proficient RNase E variants to be overproduced without causing cell death. Among the mutants that can survive overexpression, a subset appears to confer a dominant-negative phenotype; *i.e.*, these mutants interfere with

¹Corresponding author: Department of Biochemistry and Molecular Biology, University of Miami Miller School of Medicine, 1011 NW 15th St., Miami, FL 33136. E-mail: cjain@med.miami.edu

the function of wild-type RNase E. Here we describe the RNase E dominant-negative mutants that were identified, show that these mutants are capable of forming mixed multimers with wild-type RNase E *in vivo*, and propose a model to explain the basis for the dominant-negative phenotype observed.

MATERIALS AND METHODS

Plasmids and strains: Plasmid pLAC-RNE is a derivative of pACYC184 (CHANG and COHEN 1978) and pRNE401 (JAIN *et al.* 2002) that contains an *rne* gene lacking the 5'-UTR and is transcribed from a P_{lac} promoter. pNRNE5 encodes amino acids 1–498 of the RNase E N-terminal domain followed by a hexahistidine sequence (LEE *et al.* 2002). Strains WM1/F', CJ1830, and CJ1825 have been previously described (JAIN and BELASCO 1996; JAIN 2002). The mutator strain used for plasmid mutagenesis is a derivative of XL-1 Red (Stratagene, La Jolla, CA) that contains an F' *lacI^q* element to prevent overexpression of RNase E when transformed with pLAC-RNE.

Plasmid mutagenesis and screening for dominant-negative RNase E variants: pLAC-RNE was mutagenized by treatment with 50 μ g/ml of nitrosoguanidine *in vivo* for 60–150 min (MILLER 1972), by exposure to short-wave ultraviolet light (240 nm) for 30 sec on a UV transilluminator or by growth in a mutator strain. Mutagenized plasmid DNA was transformed into CJ1830 and plated on LB plates containing chloramphenicol (Cm) and X-gal. After overnight incubation at 37°, the transformed colonies were screened for the *lacZ* phenotype. Typically, 100–1000 colonies were obtained per transformation. Colonies that appeared more blue than parallel CJ1830 transformants containing control pACYC184 DNA were picked and inoculated in LB plus Cm to grow cultures for plasmid DNA preparation. The plasmid DNAs were retransformed into CJ1830 and the transformed strains were assayed for β -galactosidase activity along with CJ1830/pACYC184 strains to reconfirm and quantify the dominant-negative phenotype. Plasmids that reproducibly increased *rne-lacZ* activity in CJ1830 were sequenced.

RNA half-life analysis: RNA half-lives were measured in an isogenic pair of *E. coli* strains: CJ1825 cotransformed with pBR322, a ColE1 plasmid (BOLIVAR 1978), and either the control plasmid pACYC184 or a plasmid expressing the DNX5 dominant-negative variant (pDNX5). The transformed cells were grown to midlog phase at 37° in LB medium supplemented with Cm and ampicillin. Rifampicin (0.2 mg/ml) was added to each culture, and total cellular RNA was extracted at different time intervals thereafter. Equal amounts of each RNA sample (8 μ g) were analyzed by primer extension using a mixture of two 5'-end-labeled DNA oligonucleotides complementary to *rpsO* mRNA and RNA I, as previously described (JAIN *et al.* 2002). The primer extension products were fractionated by electrophoresis on a denaturing 4% polyacrylamide gel and quantitated using a Molecular Dynamics storm 820 phosphorimager. The physical half-lives of the two RNAs in each host strain were calculated from the slopes of semilogarithmic plots of RNA concentration *vs.* time, as determined by linear regression analysis.

RNase E purification: For RNase E protein expression and protein-protein interaction studies, plasmids containing pDNX5 or pNRNE5 were transformed separately or together into CJ1825. Transformed colonies were grown at 37° in 100 ml of LB with antibiotics to midlog phase ($OD_{600} = 0.5$) and induced with 1 mM IPTG for 2 hr prior to harvesting. Cells were resuspended in 1 ml of lysis buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5 mM imidazole, 1 mM PMSF) and lysed by freeze-thaw and sonication. Subsequently, the cell lysates were cleared by centrifugation in a microfuge at 14,000 rpm for 15 min at 4°.

The cleared lysates were incubated for 1 hr at 4° with 100 μ l of Ni-NTA beads (Novagen) and His-tagged protein purification was carried out as described (JAIN and BELASCO 1996). Proteins binding to the Ni-NTA beads were eluted with a small volume of lysis buffer containing 120 mM imidazole.

Western blot analysis: Equal amounts of cleared cell lysates or protein eluates were fractionated by SDS-PAGE and transferred to nitrocellulose membrane. The membranes were incubated with primary polyclonal rabbit anti-RNase E antibodies, followed by incubation with HRP-conjugated anti-rabbit antibody (Amersham, Arlington Heights, IL). RNase E products were detected by chemiluminescence (ECL Advanced/Amersham).

RESULTS

A selection strategy for identifying RNase E dominant-negative mutants: It has been previously shown that overproduction of RNase E is detrimental for growth, and cells that contain multicopy *rne* plasmids grow at reduced rates (CLAVERIE-MARTIN *et al.* 1991; JAIN *et al.* 2002). To buffer its expression, RNase E possesses a mechanism to autoregulate its production in the cell. This is mediated by recognition of the *rne* mRNA 5'-untranslated region (5'-UTR) by RNase E, which results in cleavage of the *rne* mRNA (JAIN and BELASCO 1995). Under conditions of RNase E excess due to random fluctuations in the cell, *rne* mRNA is cleaved faster, which limits further production of RNase E until equilibrium levels of RNase E have been established. Conversely, if RNase E is present at low concentrations in the cell, *rne* mRNA becomes more stable, resulting in increased RNase E translation until normal levels have been restored.

To investigate the consequences of unregulated RNase E production on cell viability, a multicopy *rne* plasmid lacking the 5'-UTR sequences (pLAC-RNE) was constructed. This plasmid also contains a replacement of the P_{rne} promoter with a slightly stronger P_{lac} promoter. Plasmid pLAC-RNE was transformed into strain WM1/F', which overproduces Lac repressor, to prevent prior induction of RNase E expression in the transformed cells. The transformed cells were grown to saturation in LB medium supplemented with Cm, and dilutions of the saturated cultures were grown on plates containing different levels of IPTG to induce Lac Repressor. The colonies appearing on the different plates were counted 48 hr after incubation at 37°, and the relative efficacy of plating was determined in each case (Table 1). It was observed that partial induction of Lac repressor with 1–10 μ M of IPTG had a modest effect on cell viability, but a 10⁴-fold or greater drop in cell viability was seen on plates containing 100 or 1000 μ M of IPTG. The inability of cells containing wild-type pLAC-RNE to plate efficiently when RNase E is overproduced suggested an efficient means of isolating functionally defective plasmid-borne RNase E variants. The strategy designed to identify such dominant-negative mutants is shown diagrammatically in Figure 1.

TABLE 1

Effect of IPTG on strains transformed with pLAC-RNE

IPTG concentration (μM)	No. of colonies/ml	Efficiency of plating
0	$2.0 \pm 0.1 \times 10^8$	1.0
1	$2.0 \pm 0.3 \times 10^8$	1.0
10	$1.1 \pm 0.1 \times 10^8$	0.5
100	$2.3 \pm 1.0 \times 10^4$	0.0001
1000	$4 \pm 4 \times 10^3$	0.00002

Plasmid pLAC-RNE was transformed into strain WM1/F', a strain containing an F' *lacI^h* episome, and plated on Cm plates. Cm^R transformants were grown to saturation and dilutions of the cultures were plated on Cm plates containing 0, 1, 10, 100, or 1000 μM IPTG. After 48 hr of incubation at 37°, the numbers of colonies growing on the different plates were counted and multiplied by the appropriate dilution factor. The efficiency of plating was calculated by dividing the number of colonies normalized on plates containing the indicated IPTG concentration by the number of colonies on plates lacking IPTG.

To identify such RNase E mutants, pLAC-RNE was mutagenized by passage through mutator strains, by treatment with the mutagen nitrosoguanidine, or by exposure to ultraviolet light (MATERIALS AND METHODS) and transformed into the recipient strain CJ1830. This strain contains a number of features that make it easier to identify and characterize the mutants obtained. First, CJ1830 contains a deletion of the *lac* repressor gene, so that functionally defective mutants of RNase E can be selected directly without the requirement for IPTG addition on plates. Second, the strain contains an *rne-lacZ* fusion reporter gene harbored within a λ -prophage integrated into the chromosome. This reporter, like the *rne* gene itself, is negatively regulated by RNase E, so that its expression provides a simple means to gauge the level of RNase E activity in the cell (JAIN and BELASCO 1995). Finally, to avoid the complications due to autoregulation of the *rne* gene, the P_{rne} promoter and the 5'-UTR of the chromosomal *rne* gene were replaced with the P_{lac} promoter, as described for the pLAC-RNE plasmid. In this strain, RNase E is expressed at constitutive levels, but because the *rne* 5'-UTR is absent, changes in *rne* function are expected to be more noticeable, as compared to a wild-type strain.

CJ1830 cells transformed with unmutagenized or mutagenized pLAC-RNE DNA were plated on LB plates containing Cm and X-gal, a colorimetric indicator of *lacZ* activity. Many more transformants were obtained when using mutagenized DNA, indicating that plasmid mutations increased the survival of the transformed cells. The *lacZ* phenotype of the pLAC-RNE transformants was compared with control transformants containing a non-*rne* Cm^r plasmid, pACYC184. The majority of pLAC-RNE transformants displayed a *lacZ* phenotype similar to CJ1830/pACYC184 cells, suggesting that these cells harbored pLAC-RNE variants producing nonfunc-

FIGURE 1.—Strategy used to identify RNase E dominant-negative mutants. Plasmid pLAC-RNE contains an *rne* gene (shaded rectangle) transcribed from a P_{lac} promoter. Plasmid DNA was mutagenized (MATERIALS AND METHODS) and transformed into the reporter strain CJ1830. CJ1830 contains an *rne* gene encoding wild-type RNase E transcribed from a P_{lac} promoter and a chromosomal *rne-lacZ* reporter fusion (shaded/solid rectangle) that is negatively regulated by RNase E. Because CJ1830 lacks Lac repressor, P_{lac} expression of the plasmid encoded *rne* gene is fully induced. Therefore, the expression of the wild-type RNase E protein or of mutants that retain substantial activity causes cell death. Only transformants that contain RNase E mutants that display a significant defect are able to form viable colonies (denoted below as circles). Amongst these, a subset of the mutants that confer a dominant-negative phenotype show an enhanced *lacZ* phenotype (solid circles).

tional or partially functional RNase E. In addition, a small fraction of the transformants ($\sim 1\%$) displayed a blue colony color phenotype that was significantly more intense than that of the CJ1830/pACYC184 strain. This *lacZ*-up phenotype suggested the presence of RNase E mutants that can disrupt the function of chromosomally expressed RNase E and increase *rne-lacZ* expression by acting as dominant-negative mutants. The characterization of such mutants is described below.

FIGURE 2.—RNase E dominant-negative mutants isolated in this study. (A) Predicted amino acid changes and increase in *rne-lacZ* activity for the dominant-negative mutants. The mutant designations are indicated in the first column, with the number of times each mutant was isolated denoted in brackets. The second column indicates the fold increase in β -galactosidase activity when CJ1830 cells were transformed with the corresponding dominant-negative mutant. To derive these values, the β -galactosidase activity of each CJ1830 strain expressing the indicated dominant-negative mutant was divided by the β -galactosidase activity of CJ1830 containing the control plasmid, pACYC184 (typically, 80 Miller units; MILLER 1972). Each measurement reflects the average of three independent determinations, with standard deviations typically <10%. The predicted amino acid changes for each RNase E variant is shown in the last column. (B) To confirm that the dominant-negative mutants encode truncated proteins, cell extracts derived from the strains used for the *lacZ* assays were used for Western blot analysis using anti-RNase E antibodies. The positions of the wild-type chromosomally encoded RNase E and the truncated plasmid-encoded dominant-negative mutants are indicated. The migration of the protein molecular weight markers is also shown. C, control strain containing pACYC184, a non-*rne* plasmid.

Characterization of RNase E dominant-negative mutants: Extensive screening of CJ1830 cells transformed with pLAC-RNE DNA mutagenized by different approaches led to the identification of seven different dominant-negative mutants (Figure 2A). Most of the mutants were identified several times from the same pools, indicating comprehensive coverage. To quantify the defects caused by the dominant-negative mutants, CJ1830 harboring each of the seven plasmid-encoded dominant-negative mutants or pACYC184 was assayed for β -galactosidase activity. Each of the plasmid mutants was found to increase β -galactosidase activity 2.6- to 8.6-fold, as compared to the CJ1830/pACYC184 strain. As an inverse relationship between *rne-lacZ* activity and RNase E activity has been demonstrated (JAIN *et al.* 2002), the plasmid-encoded RNase E variants are expected to reduce RNase E activity by 60–90% in the cell. It is possible that mutants that reduce RNase E activity further might have been present in the mutagenized plasmid pool, but their identification may have been precluded because too great an attenuation of RNase E activity leads to cell death (JAIN *et al.* 2002).

Sequencing analysis of the plasmid-encoded mutants was performed to identify the mutations that confer the dominant-negative phenotype. Unexpectedly, each of the mutant plasmids were found to contain *rne* mutations that either shift the translation reading frame or introduce stop codons, generating truncated RNase E variants containing between 417 and 491 *rne* amino

acids (Figure 2A). Two mutants, DNX5 and DNX11, also contained additional mutations. Given the high frequency of mutations that created premature stop codons, several nonfunctional plasmid variants that did not display a dominant-negative phenotype were also sequenced. In each case, only missense mutants were identified (data not shown), ruling out the possibility that the mutagenized libraries themselves contained a large proportion of mutants that contained premature stop codons.

To confirm that the dominant-negative mutants express truncated versions of the RNase E protein, cell extracts prepared from strains harboring the mutant plasmids were fractionated by electrophoresis and analyzed by Western blotting using RNase E antibodies (Figure 2B). In each case, a prominent band corresponding to a shortened RNase E variant was observed. Collectively, these results suggest that the dominant-negative phenotype arises primarily, if not exclusively, from mutations that result in truncated RNase E variants, rather than from those that cause amino acid substitutions.

Analysis of the dominant-negative mutants in a wild-type *rne* strain background: To further characterize the dominant-negative mutant, plasmids encoding the seven DNX mutants were transformed into CJ1825, a strain that contains a wild-type *rne* locus (JAIN and BELASCO 1995). Like CJ1830, CJ1825 contains an *rne-lacZ* fusion on a λ -prophage to enable RNase E activity to be readily assessed. Transformation of each plasmid resulted in an increase in *rne-lacZ* activity. However, the magnitude

FIGURE 3.—Expression of dominant-negative mutants in a wild-type *rne* strain background. (A) Plasmids containing the indicated mutants or a control plasmid (pACYC184) were transformed into CJ1825. The transformants were grown to midlog phase and assayed for β -galactosidase activity. The magnitude of the dominant-negative effect is the β -galactosidase activity of CJ1825 derivatives expressing the indicated dominant-negative mutant divided by the β -galactosidase activity of the same strain containing pACYC184. (B) Western blot analysis. Strain CJ1825 transformed with pACYC184 or pDNX5 was grown to midlog phase in LB plus Cm and used to prepare cell extracts. A total of 20 μ g of cell-extract protein was fractionated by SDS-PAGE and analyzed by Western blot analysis using anti-RNase E antibodies. The migration of full-length RNase E is indicated by an arrow.

of increase in this strain background (1.5- to 2.2-fold; Figure 3A) was smaller than in CJ1830 (2.6- to 8.6-fold; Figure 2A). This result is not unexpected because CJ1825 contains the *rne* 5'-UTR, which is important for RNase E autoregulation. One prediction would be that the expression of a dominant-negative RNase E variant in CJ1825 should increase chromosomal RNase E production due to slower degradation of the chromosomal *rne* mRNA. This prediction was tested by comparing RNase E levels in CJ1825 transformed with either pACYC184 or pDNX5. Western blot analysis confirmed that the levels of full-length RNase E were significantly higher in the latter strain (Figure 3B). Thus, the detrimental effect of the dominant-negative mutant on RNase E function in a wild-type strain background is partially offset by increased production of chromosomal RNase E.

Expression of the DNX5 RNase E variant increases the half-life of RNase E substrates: To ascertain directly whether the expression of the dominant-negative mutants interferes with RNase E-mediated mRNA degradation, the half-life of RNase E substrates were determined in CJ1825 containing either a control plasmid or pDNX5. Each strain was grown to midlog phase, at which point rifampicin was added to block further transcription. Samples were withdrawn at various times thereafter and RNA was prepared from the cultures. The RNA samples were hybridized with probes specific to two RNase E substrates: the *rpsO* mRNA that encodes the ribosomal protein S15 and the RNA I transcript that acts as an antisense regu-

FIGURE 4.—Effect of expressing the DNX5 RNase E variant on the half-life of *rpsO* and RNA I transcripts. CJ1825 transformed with pACYC184 or pDNX5 was grown to midlog phase, treated with rifampicin, and used to prepare total RNA from fractions harvested at different times. Equal amounts of each RNA sample were used for primer extension using *rpsO* and RNA I-specific probes and fractionated on a denaturing polyacrylamide gel alongside radiolabeled 100 base pair DNA markers (lane 1). The positions of the 85-nucleotide RNA I and the 266-nucleotide *rpsO* primer extension products are indicated. For each RNA sample used, the time (in minutes) at which the corresponding samples were harvested after rifampicin addition is indicated.

lator of ColE1 plasmid replication (LIN-CHAO and COHEN 1991; HAJNSDORF *et al.* 1996). By quantifying the amounts of *rpsO* and RNA I transcripts remaining at different times after rifampicin addition, it was found that expression of the DNX5 mutant increased the half-life of both RNAs (Figure 4). On the basis of the best-fit to first-order decay kinetics, the *rpsO* transcript was found to decay with a half-life of 2 min in cells containing the control plasmid, and 4.7 min in cells expressing the DNX5 mutant. Similarly, for RNA I, the half-life increased from 2.8 to 6.3 min. The increased half-life of the RNAs is similar to the 2.2-fold increase in the activity of *rne-lacZ* fusions observed upon transformation of CJ1825 with pDNX5 (Figure 3A). These experiments confirm that the dominant-negative mutant directly affects RNase E-mediated RNA decay.

Attempts were also made to determine the effects of the other mutants on mRNA decay. Since the other mutants have a small effect on *rne-lacZ* activity in CJ1825, the CJ1830 autoregulation-deficient strain background was used. However, for reasons that are unclear, the decay of the tested RNAs did not show a good fit to first-order kinetics, and no reliable measure of the degree of RNA stabilization imparted by the other mutants could

FIGURE 5.—Complex formation between the DNX5 RNase E mutant and the His-tagged amino-terminal domain of RNase E. Plasmids pDNX5 and pNRNE5, which encode a His-tagged amino-terminal domain of RNase E were transformed individually or together into strain CJ1825. Transformed colonies were grown in LB medium containing the appropriate antibiotics and induced with IPTG prior to harvesting. (A) Equal amounts of cell lysates prepared from these strains were fractionated by SDS-PAGE, transferred to nitrocellulose membrane, and the RNase E products were detected by Western blot analysis using anti-RNase E antibodies. (B) Each of the cell lysates was incubated with Ni-NTA beads, after which the beads were washed extensively to remove unbound protein. Thereafter, His-tagged RNase E was eluted by adding buffer containing imidazole. Equal volumes of the eluted fractions were fractionated by PAGE and analyzed by Western blot analysis.

be obtained. However, as all of the dominant-negative mutants have similar carboxy-terminal truncations, it is likely that in common with the DNX5 mutant, they too impair RNA degradation, albeit to a lesser degree.

Formation of mixed multimeric complexes *in vivo*:

Different models can be proposed to explain the basis of the dominant-negative effect by the DNX mutants (see DISCUSSION). One possibility is that expression of the dominant-negative mutants results in the formation of inactive or partially active multimeric complexes between the wild-type protein and the overexpressed mutants (HERSKOWITZ 1987). RNase E has already been shown to possess self-interaction domains using the yeast two-hybrid system (VANZO *et al.* 1998), and recently it has also been found to form multimers *in vitro* (CALLAGHAN *et al.* 2003; JIANG and BELASCO 2004). This suggested the possibility that the dominant-negative phenotype could arise due to the formation heteromeric RNase E complexes *in vivo*.

To determine whether mixed complexes between dominant-negative RNase E variants and RNase E can form *in vivo*, CJ1825 was transformed with combinations of plasmids encoding the DNX5 dominant-negative mutant and/or a His-tagged version of the N-terminal domain of RNase E (amino acids 1–498). The expression of these RNase E variants was detected by Western blotting (Figure 5A). The substantially greater abundance of the His-tagged amino-terminal domain of RNase E in comparison to the DNX5 mutant is probably due to the optimized Shine–Dalgarno sequence preceding the RNase E coding region in pNRNE5 (LEE *et al.* 2002). Full-length His-tagged RNase E was not used, since preliminary experiments indicated that the full-length protein is proteolyzed to a considerable extent

during the preparation of cell lysates. Using extracts derived from these transformed strains, the His-tagged RNase E protein was purified using Ni-NTA beads. Upon analysis of the purified fraction using anti-RNase E antibodies, it was observed that along with the His-tagged protein, the nontagged DNX5 RNase E mutant could also be coeluted (Figure 5B). A similar result was obtained when the purification was conducted under reducing conditions, indicating that the observed association between His-tagged RNase E and the DNX5 mutant was not due to inadvertent oxidative cross-linking (data not shown). These observations suggest that the dominant-negative variant can form mixed multimers with RNase E *in vivo*, leading to affinity purification of both. As controls, the purification was also carried out using extracts from cells expressing either the His-tagged RNase E N-terminal domain or the dominant-negative mutant protein alone. In neither case were bands corresponding to the smaller dominant-negative form observed (Figure 5B), ruling out the possibilities that the smaller band purified from extracts expressing both forms of RNase E was an artifact derived from breakdown of the higher molecular weight His-tagged protein or that the dominant-negative mutant has an intrinsic affinity for Ni-NTA beads. These results suggest a basis for the dominant-negative effect (see below).

DISCUSSION

Whereas the importance of RNase E in RNA degradation and processing has become abundantly clear over the past few years, many details regarding RNase E function still remain unresolved. Several aspects of RNase E function could be elucidated through mutational analysis, but so far there have been few studies devoted to generating and characterizing RNase E mutants. To expedite such studies, we have developed a scheme to generate functionally defective RNase E mutants, which is based on the effective discrimination against active RNase E variants from those that are significantly defective. During the screening process, we observed that a subset of the defective mutants conferred a dominant-negative phenotype. These mutants are the focus of the studies described here.

A priori, there is no reason to expect that dominant-negative mutants of RNase E must exist. The fact that we were able to identify such mutants, therefore, provides some clues about the manner by which RNase E recognizes and cleaves mRNAs in the cell. Furthermore, the observation that the dominant-negative mutants encode C-terminal truncated RNase E variants provides additional constraints on RNase E function *in vivo*. Similar plasmid-encoded truncated RNase E mutants were previously generated, but they were not tested for a dominant-negative phenotype (OW *et al.* 2000).

One possible mechanism by which dominant-negative mutants could exert their inhibitory effect is if they were

defective for catalysis, but remained proficient for binding mRNAs. Such mutants could manifest a dominant-negative phenotype by competing with wild-type RNase E to access the same (limiting) substrates. However, we believe that this is not a likely possibility. Our screening for dominant-negative mutants did not reveal any catalytically inactive missense mutants, even though, on the basis of our current understanding of ribonucleases, such inactive mutants should exist. This suggests that inactive missense mutants do not confer a dominant-negative phenotype. Supporting this notion, we have identified a number of RNase E missense mutants that are functionally defective and that are expressed at high levels in the cell, but do not confer a dominant-negative phenotype (K. BRIEGEL and C. JAIN, unpublished data). A second possibility is that the dominant-negative mutants could sequester limiting host factors that are necessary for RNase E function. Although RNase E is known to bind a number of proteins, notably the components of the degradosome, these associations require the carboxy-terminal domain of RNase E. It is unclear how expression of the dominant-negative RNase E variants that lack the carboxy-terminal domain could interfere with the interactions between those proteins and wild-type RNase E.

A third possibility, which we favor, is that mixed multimeric complexes between wild-type RNase E and the dominant mutants can form and that this association inhibits RNase E function. By coexpression of a tagged version of RNase E and a dominant-negative mutant, we show that such mixed complexes do form *in vivo* (Figure 5). Moreover, RNase E has been shown to possess self-interaction domains by two-hybrid analysis and to form tetramers *in vitro* (VANZO *et al.* 1998; CALLAGHAN *et al.* 2003). This raises the question—Why should the formation of mixed complexes between the dominant-negative mutants and wild-type RNase E lead to inhibition of the latter?

In principle, this inhibition could be due to the requirement of two or more monomers in an RNase E multimeric complex to either form a joint active site for RNA catalysis or form a joint RNA-binding surface necessary to recruit substrates to the active site. We believe that the former possibility is not very likely, as no missense mutants that lack catalytic function and confer a dominant-negative phenotype were found. This suggests that RNA cleavage is probably achieved by individual RNase E subunits, independent of their association with other RNase E molecules. A more likely explanation is that two or more monomers in a multimeric complex are required to bind RNA effectively and that loss of the RNA-binding determinants on one or more RNase E monomers reduces the efficacy of RNA recognition (Figure 6). A substantial test of this hypothesis will require the technically challenging purification of mixed multimers of wild-type and dominant-negative mutants and comparing the RNA-binding and cleavage

FIGURE 6.—Model for the dominant-negative phenotype conferred by truncated RNase E variants. RNase E is proposed to bind RNA as a multimer, with two or more RNase E monomers in this complex contributing to RNA binding. For simplicity, RNase E is shown as a dimer, and only the catalytic domain and RNA-binding domains are depicted schematically. The recruitment of the RNA substrates to the enzyme-active site leads to RNA cleavage (left). When truncated dominant-negative mutants of RNase E lacking the RNA-binding domains are expressed, they form mixed multimers with wild-type RNase E, which are unable to bind RNA substrates efficiently. Hence, the rate of cleavage of mRNA substrates is reduced in the cell (right).

properties of these hetero-multimeric complexes with the homo-multimeric wild-type RNase E complexes.

This hypothesis raises the question of why missense mutants that interfere with RNA binding could not be identified in the screen for dominant-negative mutants. One possible explanation is that RNase E possesses multiple or degenerate RNA-binding domains, so that mutating any one of these sites may not result in significant impairment of RNA binding. That RNase E contains multiple RNA-binding sites can be inferred from the fact that deletion mutants lacking the central arginine-rich domain of RNase E, which displays robust RNA-binding affinity *in vitro*, are nonetheless able to cleave RNA (TARASEVICIENE *et al.* 1995; McDOWALL and COHEN 1996). Therefore, the most straightforward way to generate an RNase E mutant defective for RNA binding would be to introduce stop codons upstream of the degenerate RNA-binding domains, resulting in truncated variants that retain the ability to form mixed dimers but are unable to bind RNA efficiently.

In this context, it is worth noting that the preference that RNase E displays for cleaving substrates containing a 5' monophosphate rather than a 5' triphosphate or a hydroxyl group also appears to be dependent upon the ability of RNase E to form multimers (JIANG and BELASCO 2004). This suggests that a joint surface formed by an RNase E multimeric complex may also be necessary for RNA 5'-end discrimination. Whether the same surface recognizes the RNA 5'-end and the bulk of the RNA substrate, as we propose here, remains to be determined.

Interestingly, RNase E mutants containing as few as 402 N-terminal amino acids retain RNA cleavage activity, albeit to a reduced degree as compared with longer forms of RNase E (NILSSON *et al.* 2002). Three of the dominant-negative mutants (DNX21, DNX23, and DNX25) were

also found to be able to complement the defect in a strain background harboring a chromosomal *rne-1* temperature-sensitive allele at the nonpermissive temperature, indicating sufficient *in vivo* activity for cell survival (data not shown). These results suggest that the mixed multimers may retain some RNase activity. The degradation of RNAs in cells expressing the dominant-negative mutants would then be mediated by a combination of the residual activity of mixed multimers containing wild-type and mutant forms of RNase E, small amounts of wild-type homo-multimers, and other cellular ribonucleases.

Dominant-negative mutants have been used extensively to inactivate gene function. One way to regulate such inactivation is by transcribing such mutants from an inducible promoter. Upon induction of the promoter, the dominant-negative mutant can be rapidly synthesized to exert a negative effect on gene function. With the identification of RNase E dominant-negative mutants, a similar principle could also be applied to RNase E. At present, the most common method to inactivate RNase E is by using temperature-sensitive mutants. However, such mutants cannot be used in many situations, and their use can also lead to unintended consequences, such as the induction of heat-shock response. The alternate strategy, to use RNase E dominant-negative mutants expressed from a regulated promoter, could become a useful alternative for studying the *in vivo* functions of RNase E in the future.

We thank Kangseok Lee, Stanley Cohen, and Rik Myers for strains or plasmids and Joel Belasco, Murray Deutscher, and George Mackie for valuable comments on the manuscript. These studies were supported by start-up funds from the Lucille P. Markey Foundation.

LITERATURE CITED

- BOLIVAR, F., 1978 Construction and characterization of new cloning vehicles. III. Derivatives of plasmid pBR322 carrying unique Eco RI sites for selection of Eco RI generated recombinant DNA molecules. *Gene* **4**: 121–136.
- BOUVET, P., and J. G. BELASCO, 1992 Control of RNase E-mediated RNA degradation by 5'-terminal base pairing in *E. coli*. *Nature* **360**: 488–491.
- BYCROFT, M., T. J. HUBBARD, M. PROCTOR, S. M. FREUND and A. G. MURZIN, 1997 The solution structure of the S1 RNA binding domain: a member of an ancient nucleic acid-binding fold. *Cell* **88**: 235–242.
- CALLAGHAN, A. J., J. G. GROSSMANN, Y. U. REDKO, L. L. ILAG, M. C. MONCRIEFFE *et al.*, 2003 Quaternary structure and catalytic activity of the *Escherichia coli* ribonuclease E amino-terminal catalytic domain. *Biochemistry* **42**: 13848–13855.
- CARPOUSIS, A. J., G. VAN HOUWE, C. EHRETSMANN and H. M. KRISCH, 1994 Co-purification 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.
- CHANG, A. C., and S. N. COHEN, 1978 Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.* **134**: 1141–1156.
- CLAVERIE-MARTIN, F., M. R. DIAZ-TORRES, S. D. YANCEY and S. R. KUSHNER, 1991 Analysis of the altered mRNA stability (*ams*) gene from *Escherichia coli*. *J. Biol. Chem.* **266**: 2843–2851.
- 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.
- CONDON, C., and H. PUTZER, 2002 The phylogenetic distribution of bacterial ribonucleases. *Nucleic Acids Res.* **30**: 5339–5346.
- DEUTSCHER, M. P., and Z. LI, 2001 Exoribonucleases and their multiple roles in RNA metabolism. *Prog. Nucleic Acid Res. Mol. Biol.* **66**: 67–105.
- 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.
- HAJNSDORF, E., F. BRAUN, J. HAUGEL-NIELSEN, J. LE DEROUT and P. REGNIER, 1996 Multiple degradation pathways of the rpsO mRNA of *Escherichia coli*. RNase E interacts with the 5' and 3' extremities of the primary transcript. *Biochimie* **78**: 416–424.
- HERSKOWITZ, I., 1987 Functional inactivation of genes by dominant negative mutations. *Nature* **329**: 219–222.
- JAIN, C., 2002 Degradation of mRNA in *Escherichia coli*. *IUBMB Life* **54**: 315–321.
- JAIN, C., and J. G. BELASCO, 1995 RNase E autoregulates its synthesis by controlling the degradation rate of its own mRNA in *Escherichia coli*: unusual sensitivity of the *rne* transcript to RNase E activity. *Genes Dev.* **9**: 84–96.
- JAIN, C., and J. G. BELASCO, 1996 A structural model for the HIV-1 Rev-RRE complex deduced from altered-specificity Rev variants isolated by a rapid genetic strategy. *Cell* **87**: 115–125.
- 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.
- 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.
- 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 RNA I regulates the replication of ColEI-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.
- MACKIE, G. A., 1998 Ribonuclease E is a 5'-end-dependent endonuclease. *Nature* **395**: 720–723.
- 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., and S. N. COHEN, 1996 The N-terminal domain of the *rne* gene product has RNase E activity and is non-overlapping with the arginine-rich RNA-binding site. *J. Mol. Biol.* **255**: 349–355.
- MCDOWALL, K. J., S. LIN-CHAO and S. N. COHEN, 1994 A+U content rather than a particular nucleotide order determines the specificity of RNase E cleavage. *J. Biol. Chem.* **269**: 10790–10796.
- 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.
- MILLER, J. H., 1972 *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- NILSSON, R. P., A. VON GABAIN and V. R. KABERDIN, 2002 In-gel analysis of site-specific rnases. *Biotechniques* **33**: 272, 274, 276.
- 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 DEAD-box RNA helicase in the *Escherichia coli* RNA degradosome. *Nature* **381**: 169–172.
- 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.* **341**: 37–54.
- STEEGE, D. A., 2000 Emerging features of mRNA decay in bacteria. *RNA* **6**: 1079–1090.
- 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.

Communicating editor: S. GOTTESMAN