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

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RNase E is a key regulatory enzyme that appears to control the principal pathway for mRNA degradation in *Escherichia coli*. Here, we show that RNase E represses its own synthesis by reducing the cellular concentration of the *rne* (RNase E) gene transcript. Autoregulation is achieved by modulating the longevity of this 3.6-kb mRNA, whose half-life ranges from <40 sec to >8 min depending on the level of RNase E activity in the cell. Feedback regulation is mediated in *cis* by the 5'-terminal 0.44-kb segment of *rne* mRNA, which is sufficient to confer this property onto a heterologous transcript to which it is fused. Like the intact protein, an amino-terminal fragment of RNase E lacking 563 amino acid residues can act in *trans* to repress *rne* gene expression. Paradoxically, raising the *rne* gene copy number 21-fold in *E. coli* causes an unexpected reduction in the concentration of the full-length *rne* transcript, yet results in a small increase in RNase E protein production. These surprising phenomena are explained in terms of a model in which the degradation of this long and highly labile mRNA commences before elongation of the nascent transcript has been completed. In such circumstances, gene expression can be unusually sensitive to changes in mRNA stability.

[**Key Words:** RNase E; *rne*; mRNA degradation; gene regulation; *Escherichia coli*]

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Messenger RNA (mRNA) degradation is an important mechanism for regulating gene expression in all organisms. The steady-state concentration of a message is as sensitive to its rate of decay as it is to its rate of synthesis, and rates of mRNA decay can differ considerably within a single cell. In *Escherichia coli*, for example, mRNA half-lives can be as short as a fraction of a minute or as long as half an hour (Belasco 1993). Through their influence on mRNA abundance, these half-life differences have a corresponding effect on protein synthesis (Klug et al. 1987; Newbury et al. 1987).

An important advance in understanding the mechanism of prokaryotic mRNA degradation was the identification of RNase E as a key ribonuclease controlling mRNA decay in *E. coli*. Cleavage by this endonuclease appears to be a critical step in the degradation of most *E. coli* mRNAs, as the decay rate of bulk mRNA and of many individual transcripts slows when RNase E is thermally inactivated in *E. coli* strains with a temperature-sensitive mutation in the structural gene for this enzyme (the *rne* gene, also known as the *ams* or *hmp1* gene) (Apirion 1978; Ono and Kuwano 1979; Mudd et al. 1990; Babitzke and Kushner 1991; Melefors and von Gabain 1991; Taraseviciene et al. 1991). Although RNase E can

form a complex with at least three other *E. coli* proteins, it has been shown that *rne*-dependent endonuclease activity resides in the 118-kD RNase E polypeptide per se (L.-H. Chen and J.G. Belasco, unpubl.; Cormack et al. 1993; Carpousis et al. 1994). As yet, the RNA structural features that define the cleavage site specificity of this enzyme are not well understood (Ehretsmann et al. 1992; Lin-Chao et al. 1994; McDowall et al. 1994).

RNase E appears to be an essential *E. coli* gene product, as all *rne^{ts}* mutants that are currently available display a temperature-sensitive lethal phenotype (Apirion 1978; Ono and Kuwano 1979). Furthermore, overexpression of the *rne* gene on high-copy plasmids slows cell growth and can lead to plasmid loss or the acquisition of *rne* mutations (Claverie-Martin et al. 1991; C. Jain and J.G. Belasco, unpubl.). Given the importance of RNase E for mRNA metabolism in *E. coli* and the apparent sensitivity of cells to levels of RNase E activity that are excessively high or low, it would seem reasonable to expect that *E. coli* might have evolved a mechanism for tightly regulating the production of this key ribonuclease. Consistent with this hypothesis, a temperature-sensitive RNase E variant and its cognate mRNA each accumulate to a higher cellular concentration when this

enzyme is thermally inactivated at 44°C, and this increase in the abundance of the full-length *rne* transcript can be reversed by introducing a plasmid clone of a truncated *rne* gene that is functional at this temperature [Melfors 1991; Mudd and Higgins 1993].

In this study we present evidence that RNase E regulates its own synthesis by controlling the decay rate of *rne* mRNA, whose half-life can vary by more than a factor of 14 in response to changes in cellular RNase E activity. By deletion mapping, we have identified the domains of the *rne* transcript and of RNase E that mediate this autoregulatory phenomenon. Our data suggest that the unusual sensitivity of *rne* gene expression to cellular RNase E activity is the result, in part, of cotranscriptional degradation of nascent *rne* transcripts, a process that can amplify the impact of mRNA stability changes by influencing both the production and decay of mRNA templates for RNase E synthesis.

Results

rne-lacZ fusions are unusually responsive to RNase E activity in vivo

To assess the ability of RNase E to regulate *rne* gene expression, we first constructed a translational fusion in which the promoter, 5'-untranslated region (UTR), and first 181 codons of the *rne* gene were joined in-frame to *lacZ*, and we introduced a single copy of this fusion (*ez1*) as a λ lysogen into the chromosome of a *lacZ*⁻ *E. coli* strain. We then determined the effect of RNase E overproduction on expression of the *rne-lacZ* fusion by comparing β -galactosidase production from the fusion in *E. coli* cells containing a single, chromosomal copy of the wild-type *rne* gene versus cells containing a multicopy plasmid clone of the *rne* gene (pRNE101, a derivative of pACYC177). Overproduction of RNase E in this manner reduces expression of the *rne-lacZ* fusion by a factor of

four (Table 1). Furthermore, expression of this gene fusion is increased when cellular RNase E activity is reduced to below-normal levels by replacing the chromosomal copy of the *rne* gene with either of two temperature-sensitive *rne* alleles (*rne-3071* or *ams-1*). For example, when a thermolabile form of RNase E is partially inactivated at 37°C in *ams-1* cells, expression of the *rne-lacZ* fusion increases ninefold to a level that is ~33 times higher than in the same cells containing a multicopy plasmid clone of the wild-type *rne* gene. In contrast, RNase E overproduction has almost no effect on the expression of the wild-type *lacZ* gene, a finding that implies that the responsiveness of the *rne-lacZ* fusion to RNase E overproduction is attributable to its *rne* component. Together, these data indicate that RNase E can repress the expression of its own gene and that a promoter-proximal *rne* gene fragment can confer this sensitivity to RNase E overproduction upon an otherwise unresponsive gene to which it is fused. Furthermore, the insensitivity of *lacZ* and two other *lacZ* gene fusions (*ompA-lacZ* and *IS10-lacZ*) to RNase E overproduction suggests that the *rne* gene is unusual in its ability to be repressed by elevated cellular levels of this ribonuclease.

rne and *rne-lacZ* transcript levels are dependent on RNase E activity in vivo

To determine whether the effect of the three *rne* alleles (wild-type, *rne-3071*, and *ams-1*) on expression of the unfused *rne* gene parallels their effect on expression of the *rne-lacZ* fusion, total cellular RNA was isolated from isogenic *rne*⁺, *rne-3071*, and *ams-1* strains containing an *rne-lacZ* fusion and analyzed by Northern blotting. Two distinct mRNAs were detected by probing the blot with radiolabeled *rne* DNA: the 3.6-kb transcript of the *rne* gene and the 4.0-kb transcript of the *rne-lacZ* fusion

Table 1. Effect of cellular RNase E activity on the expression of *rne-lacZ*, *lacZ*, and other *lacZ* fusions

Gene fusion	Chromosomal <i>rne</i> allele	β -Galactosidase activity (units) ^a		Repression ratio ^b
		pACYC177	pRNE101	
<i>rne-lacZ</i>	<i>rne</i> ⁺	172 $\pm$ 7	47 $\pm$ 3	3.7
<i>rne-lacZ</i>	<i>rne-3071</i>	591 $\pm$ 18	45 $\pm$ 1	13
<i>rne-lacZ</i>	<i>ams-1</i>	1574 $\pm$ 23	48 $\pm$ 1	33
<i>lacZ</i>	<i>ams-1</i>	2430 $\pm$ 120	3120 $\pm$ 50	0.78
<i>ompA-lacZ</i>	<i>ams-1</i>	84 $\pm$ 2	87 $\pm$ 3	0.97
<i>IS10-lacZ</i>	<i>ams-1</i>	3.8 $\pm$ 0.1	6.5 $\pm$ 0.5	0.58

^aAll β -galactosidase assays were performed on extracts of *E. coli* strains that contained either a multicopy *rne*⁺ plasmid (pRNE101) or an otherwise identical plasmid lacking an *rne* insert (pACYC177). The *lacZ*⁻ host strains for measuring expression of the *rne-lacZ* fusion were CH1827 (*rne*⁺), CH1826 (*rne-3071*), and CH1828 (*ams-1*) [Mudd et al. 1990]. The host strain for measuring expression of the *ompA-lacZ* and *IS10-lacZ* fusions was CH1828. Each fusion was introduced in single copy by lysogeny of a λ transducing phage. Wild-type *lacZ* expression was measured in HAK117 cells (*ams-1 lacZ*⁺) [Ono and Kuwano 1979] induced with IPTG (1 mM). Extracts were prepared from log-phase cultures grown at 37°C in minimal A medium [Miller 1972]. At 30°C, expression of the *rne-lacZ* fusion was 1.7-fold higher in the *rne-3071* strain and 4.4-fold higher in the *ams-1* strain compared with the *rne*⁺ strain. Each value for β -galactosidase activity represents the average and standard deviation of three measurements.

^bTo calculate repression ratios, the β -galactosidase activity in cells containing pACYC177 was divided by the β -galactosidase activity in cells containing pRNE101.

(Fig. 1). These mRNAs are barely detectable in RNA samples isolated from *rne*⁺ cultures incubated at either 37°C or 42°C. In contrast, strong bands of approximately equal intensity are apparent in lanes containing RNA isolated from the temperature-sensitive *rne-3071* and *ams-1* strains incubated at 42°C. In cultures grown at 37°C, the two mutant *rne* alleles display dissimilar effects: The *rne-3071* mutant behaves like the wild-type strain, whereas the *ams-1* mutation, which has a more severe phenotype, causes elevated transcript levels similar to those observed with the same mutation at 42°C. The differential effect of the two mutant *rne* alleles at 37°C parallels their effect on β -galactosidase production from the *rne-lacZ* fusion (Table 1). An advantage of analyzing the *ams-1* strain at 37°C is that a marked reduction in RNase E activity can be achieved without significantly impairing bacterial growth, thereby allowing the effects of this mutation to be evaluated under physiologically defined conditions. In contrast, growth of *ams-1* and *rne-3071* cells ceases after a temperature shift to 42°C; consequently, the effects of RNase E inactivation may be difficult to distinguish from the effects of growth arrest at this temperature.

These findings indicate that RNase E regulates its own synthesis by modulating the cellular concentration of the *rne* transcript. Furthermore, the similar response of *rne* mRNA and the hybrid *rne-lacZ* transcript to changes in cellular RNase E activity implies that the regulation of the *rne-lacZ* fusion by RNase E accurately mimics autoregulation of the *rne* gene and that the *rne* gene fragment present in the fusion contains all of the *cis*-acting signals necessary to mediate this phenomenon.

Figure 1. Northern blot analysis of *rne* and *rne-lacZ* transcripts in *E. coli* strains with various *rne* alleles. Total cellular RNA was isolated in triplicate from strains CJ1827 (*rne*⁺), CJ1826 (*rne-3071*), and CJ1828 (*ams-1*) that had been grown overnight at 30°C, subcultured into fresh medium at 37°C for 1 hr, and incubated for an additional hour at 37°C or 42°C prior to extracting RNA. The RNA samples (10 μ g) were fractionated by electrophoresis on a 1% agarose/0.22 M formaldehyde gel, blotted onto a nylon membrane, and probed with radiolabeled *rne* DNA. On the basis of their migration relative to rRNA and RNA molecular mass standards, the sizes of the hybridizing RNAs are close to the expected sizes of the *rne* and *rne-lacZ* transcripts (3.6 and 4.0 kb, respectively) (Claverie-Martin et al. 1991; Casarégola et al. 1992; Yarchuk et al. 1992).

Autoregulation does not involve changes in transcription

In principle, feedback regulation of the cellular concentration of the *rne* transcript could be achieved by modulating either the transcription of the *rne* gene or the decay of the *rne* transcript. To distinguish these two possibilities, we first inserted an inducible *lac* promoter (*P*_{lac}) 0.2 kb upstream of an *rne* gene fragment that contains the promoter (*P*_{rne}), 5' UTR, and the first 181 codons of the *rne* gene. The resulting plasmid, pLAC-RNE', was cotransformed into isogenic *rne*⁺ and *ams-1* *E. coli* cells along with a compatible *lacI*^r plasmid to repress *P*_{lac} transcription. RNA was isolated from these strains in the presence or absence of the *P*_{lac} inducer IPTG, and these RNA samples were subjected to primer extension analysis using a 5'-end-labeled oligonucleotide complementary to the *rne* transcript.

Major primer extension products of the sizes predicted for the *P*_{lac} and *P*_{rne} transcripts were observed (Fig. 2). As expected, the abundance of the *P*_{lac} transcript increases upon addition of IPTG, whereas the *P*_{rne} transcript is not affected by this inducer. Furthermore, the concentrations of the *P*_{lac} transcript and the *P*_{rne} transcript both increase about fourfold in the *ams-1* strain. This increase in *P*_{lac} transcript concentration would not be expected if *rne* autoregulation occurred at the level of transcription, as transcription from the *lac* promoter normally is unresponsive to RNase E overproduction (Table 1) and as the *lac* promoter is located so far upstream from *P*_{rne} that it is unlikely that any *cis*-acting *P*_{rne} element would influence transcription from *P*_{lac}. Instead, it seems likely that both the *P*_{lac} and *P*_{rne} transcripts share within their common transcribed region an *rne* mRNA determinant that mediates RNase E-dependent mRNA degradation and feedback regulation of *rne* gene expression.

Interestingly, we also observed a prominent primer extension product ~50 nucleotides shorter than that derived from the *P*_{rne} transcript. A similar truncated RNA is detected when *rne* or *rne-lacZ* mRNA is analyzed using an RNase or S1 protection assay (see below) (Claverie-Martin et al. 1991). As the abundance of the truncated RNA is increased by IPTG induction of transcription from the upstream *lac* promoter, we conclude that this RNA species must arise by ribonuclease cleavage of the *P*_{lac} and *P*_{rne} transcripts at a site within the *rne* 5' UTR and that it is not a direct product of transcription from a hypothetical promoter downstream of *P*_{rne}.

The stability of the *rne* transcript is affected by RNase E activity in *E. coli*

The arguments presented above suggest that the likely mechanism of *rne* autoregulation involves RNase E-mediated modulation of *rne* mRNA stability. To test this possibility directly, the half-life of the *rne* transcript was compared in three isogenic strains that contain different levels of RNase E activity. These strains have either an *ams-1* mutation (low RNase E activity), a wild-type *rne* gene (normal RNase E activity), or a chromosomal *rne*⁺

Figure 2. Primer extension comparison of hybrid *rne* mRNAs transcribed from the *rne* promoter and an upstream *lac* promoter. Plasmid pLAC-RNE' was transformed into *E. coli* strains CH1827 (*rne*⁺) and CH1828 (*ams-1*) containing the F-derived *lacI*^q plasmid pRJM386. Transformants were grown overnight at 30°C and subcultured for 45 min in prewarmed medium at 37°C in duplicate. IPTG (1 mM) was added to one of each duplicate culture to induce transcription from the *lac* promoter, and total cellular RNA was harvested 45 min later. Equal amounts of each RNA sample (5 µg) were analyzed by primer extension with a 5'-end-labeled oligonucleotide (5'-TAACTGCCTGAAAGATCA-3') complementary to the plasmid-encoded transcripts. The primer extension products were fractionated by electrophoresis on a 4% acrylamide/8 M urea sequencing gel beside radiolabeled ϕ X174/*Hae*III markers (not shown). Bands corresponding to *rne* mRNAs initiated at the *P*_{lac} and *P*_{rne} promoters are indicated, as is a band that corresponds to a major cleavage product of *rne* mRNA. Relative to the ϕ X174/*Hae*III markers, the *P*_{lac} and *P*_{rne} primer extension products migrate as expected based on their calculated lengths. Several additional bands of intermediate mobility are also visible, which presumably result either from cleavage of the *P*_{lac}-derived *rne* transcript in vivo or from premature termination by reverse transcriptase. The data at right were obtained by integrating the intensity of the bands corresponding to the *P*_{lac} and *P*_{rne} transcripts and the major cleavage product and normalizing each to its value in the sample from uninduced *rne*⁺ cells.

gene plus the multicopy *rne* plasmid pRNE101 (high RNase E activity). Each of these strains also contains a chromosomal *rne-lacZ* fusion. To allow the *rne* transcript to be detected in cells with a high level of RNase E activity, a sensitive RNase protection assay was used. Total cellular RNA was isolated from each of the three strains at time intervals after transcription inhibition with rifampicin, annealed with a mixture of two radiolabeled RNA probes complementary to the 5' and 3' regions of the *rne* transcript, and treated with RNase T1. The protected probe fragments were then fractionated by electrophoresis on a nondenaturing polyacrylamide gel and detected by autoradiography (Fig. 3).

Five RNase-resistant products were observed: 1.26- and 0.55-kb fragments corresponding, respectively, to the 5' and 3' segments of the *rne* transcript, a 1.21-kb fragment corresponding to a cleavage product of the *rne* transcript (see below), a 0.91-kb fragment corresponding to the 5' segment of the *rne-lacZ* transcript, and a 0.86-kb fragment corresponding to an *rne-lacZ* cleavage product. Quantification of the rate of disappearance of each band allowed the half-lives of the various mRNA segments to be calculated (Table 2). In addition, the half-life of the entire *rne* transcript in the wild-type and *ams-1* strains was measured using a long RNA probe complementary to virtually the whole 3.6-kb transcript (Table 2). The half-life of the entire *rne* transcript in cells containing pRNE101 could not be determined (see below).

In each of the three strains, the half-lives of the 5' and 3' *rne* mRNA segments are similar to one another and to the half-life of the 5' segment of the *rne-lacZ* transcript: ~9 min in the *ams-1* strain, 1 min in the wild-type strain, and 0.6 min in the overproducing strain containing pRNE101. Moreover, in the wild-type and *ams-1* strains, these half-lives are also similar to that measured for the entire *rne* transcript. Clearly, the longevity of the *rne* transcript is dependent on the level of RNase E activity in these cells. The simplest interpretation of this observation is that it reflects a modulation of the rate of RNase E-mediated cleavage of the *rne* transcript, though an indirect mechanism for the observed changes in *rne* mRNA stability cannot be excluded. In any event, these findings indicate that RNase E regulates its own synthesis by controlling the decay rate of the *rne* transcript.

Feedback repression of RNase E synthesis

The finding that the stability of the *rne* transcript and the expression of an *rne-lacZ* fusion are dependent on the level of RNase E activity in *E. coli* suggests that RNase E production should be autoregulated. To test directly whether RNase E represses its own synthesis, we compared the cellular concentration of RNase E in three isogenic strains that contain different levels of RNase E activity: an *ams-1* strain (low RNase E activity), an *rne*⁺ strain (normal activity), and an *rne*⁺ strain containing a plasmid (pRNE102) that encodes an active RNase E variant with a carboxy-terminal deletion (high activity). Each of these strains also contains a chromosomal *rne*-

Figure 3. Determination of *rne* and *rne-lacZ* transcript half-lives in *E. coli* strains with various *rne* alleles and *rne* copy numbers. Cultures of CJ1828 (*ams-1*), CJ1827 (*rne*⁺), or CJ1827 containing plasmid pRNE101 (multicopy *rne*⁺) were grown to log phase at 37°C and treated with rifampicin to inhibit transcription. Total cellular RNA was isolated at time intervals thereafter. Equal amounts of each RNA sample (5 µg) were analyzed by ribonuclease protection using a mixture of two radiolabeled RNA probes complementary to nucleotides 1–1257 and 2890–3466 of the *rne* transcript. The resulting RNase-resistant duplexes were electrophoresed on a nondenaturing 4% polyacrylamide gel beside radiolabeled φX174/*Hae*III DNA markers (not shown; see calibration). Bands corresponding to 5' and 3' segments of the *rne* transcript (1.26 and 0.58 kb, respectively) and to a 5' segment of the *rne-lacZ* transcript *ez1* (0.91 kb) are indicated. Just beneath the 5' *rne* and *rne-lacZ* bands are two additional bands (1.21 and 0.86 kb, respectively) representing products of cleavage within the identical 5' UTRs of these two mRNAs. The ribonuclease-resistant RNA duplexes migrate somewhat more slowly than DNA duplexes of the same length.

lacZ fusion, which serves as an independent reporter of feedback regulation.

Protein extracts prepared from each strain were fractionated by SDS-PAGE and analyzed by Western blotting

Table 2. Effect of cellular RNase E activity on *rne* and *rne-lacZ* mRNA stability

<i>rne</i> genotype	Half-life (min) ^a			
	<i>rne</i> full-length	<i>rne</i> 5' segment	<i>rne</i> 3' segment	<i>rne-lacZ</i> (5' segment)
<i>ams-1</i>	9.0	7.6	9.6	6.7
<i>rne</i> ⁺	1.0	0.8	1.3	1.0
pRNE101	—	0.6	0.7	0.6

^a*E. coli* strains CJ1828 (*ams-1*), CJ1827 (*rne*⁺), and CJ1827 containing plasmid pRNE101 (multicopy *rne*⁺) were grown to log phase at 37°C. Transcription was inhibited with rifampicin, and total cellular RNA was isolated at time intervals thereafter. Equal amounts of each RNA sample were analyzed by RNase protection either with a probe complementary to virtually the entire *rne* transcript (full-length) or with a pair of probes complementary to the 5' and 3' segments of *rne* mRNA (see Fig. 3). Besides revealing the half-life of *rne* mRNA and segments thereof, these experiments also provided information about the decay rate of the 5'-terminal 0.91-kb segment of the *rne-lacZ* transcript *ez1*, which is complementary to the full-length and 5' *rne* probes. The half-life of full-length *rne* mRNA in CJ1827 containing pRNE101 could not be measured because its concentration in these cells is below detectable levels. Each half-life value represents the average of two to four independent decay rate measurements.

using antibodies raised against purified RNase E (Fig. 4). The yield of the chromosomally encoded RNase E protein was found to vary inversely with cellular RNase E activity. Compared with wild-type cells, the concentration of full-length RNase E increases fivefold in *ams-1* cells and falls by almost a factor of 7 in wild-type cells containing pRNE102. The increased RNase E production in *ams-1* cells versus *rne*⁺ cells is accompanied by a similar increase in β-galactosidase synthesis from the resident *rne-lacZ* fusion (Fig. 4). Despite the greater concentration of RNase E in the *ams-1* strain, the low specific activity of the thermolabile enzyme encoded by the *ams-1* allele results in a net reduction in RNase E activity in these cells, as reflected by the increased stability of the *rne* transcript and the enhanced expression of the *rne-lacZ* fusion.

To assess how tightly the *rne* gene is autoregulated, we then quantitated the effectiveness of this feedback regulatory mechanism in compensating for an increased *rne* gene dosage. An *rne*⁺ *E. coli* strain containing a chromosomal *rne-lacZ* fusion was transformed with either of two multicopy plasmids bearing the wild-type *rne* gene: pRNE101, a derivative of pACYC177 that contains a p15A replication origin, and pRNE201, which contains a pSC101 replication origin. Western blot analysis of protein extracts showed that despite the increased *rne* gene dosage, the RNase E protein concentration in these cells is only two- to threefold higher than in the same cells lacking a plasmid (Fig. 4).

To quantify the increased *rne* gene dosage in cells containing pRNE101, the same host strain was transformed with plasmid pRNE/EZ101, which contains both an *rne*

Figure 4. Comparison of RNase E protein levels in *E. coli* strains with various *rne* alleles and *rne* copy numbers. (Left) Protein extracts were prepared from log-phase cultures of three *E. coli* strains growing at 37°C: CJ1828 (*ams-1*), CJ1827 (*rne*⁺), and CJ1827 containing plasmid pRNE102, which encodes a truncated but active form of RNase E. (Right) Protein extracts were prepared from log-phase cultures of strain CJ1827 (*rne*⁺) containing plasmid pACYC177, pRNE201, pRNE101, or pRNE/EZ101 and growing at 37°C. Equal amounts of each protein sample (5 μg) were analyzed by Western blotting, using anti-RNase E antibodies and enhanced chemiluminescent detection. The full-length RNase E protein (118 kD) and the truncated RNase E variant encoded by pRNE102 (RNase EΔ, 90 kD) migrate anomalously with apparent molecular masses of 180 and 110 kD, respectively (Claverie-Martin et al. 1989; Casarégola et al. 1992). Beneath each lane is listed the relative abundance of the full-length RNase E protein, as determined by densitometry, and the relative amount of *rne-lacZ*-derived β-galactosidase activity present in each strain.

gene and an *rne-lacZ* gene fusion and which, like pRNE101, is a derivative of pACYC177. As expected, cells containing pRNE/EZ101 produce the same somewhat elevated amount of RNase E as cells containing pRNE101 (2.6- vs. 2.8-fold; Fig. 4). By comparing β-galactosidase production from the single, chromosomal copy of the *rne-lacZ* fusion in cells containing pRNE101 and from the multiple copies of this fusion in cells containing pRNE/EZ101 (Fig. 4), we were able to calculate that the effective copy number of these two plasmids is 20. This effective copy number reflects both the physical copy number of these plasmids and any possible differences in *rne* promoter activity on the plasmid versus the *E. coli* chromosome, and it provides an estimate of the increased rate of *rne* gene transcription in cells containing pRNE101 or pRNE/EZ101. As a 21-fold increase in the effective *rne* gene copy number results in only an ~2.7-fold increase in the concentration of the RNase E protein, we calculate that the yield of RNase E per effective *rne* gene copy is reduced by a factor of 8 in cells containing pRNE101 or pRNE/EZ101. These findings demonstrate that RNase E is subject to tight, if imperfect, autoregulation.

Increasing the *rne* gene dosage reduces the cellular concentration of full-length *rne* mRNA

To determine whether the level of autoregulated RNase

E production is correlated with the steady-state concentration of the full-length *rne* transcript, RNA from an *ams-1* strain, an *rne*⁺ strain, and an *rne*⁺ strain containing plasmid pRNE101 was analyzed by RNase protection using a radiolabeled probe complementary to the entire *rne* transcript (Fig. 5). Consistent with the Northern blot shown in Figure 1, the concentration of full-length *rne* mRNA in wild-type cells is only 5–10% of that in *ams-1* cells, which contain a thermolabile form of RNase E with reduced specific activity. Interestingly, no full-length *rne* mRNA can be detected in cells that contain

Figure 5. Steady-state levels of the full-length *rne* transcript in *E. coli* strains with various *rne* alleles and *rne* copy numbers. Total cellular RNA was isolated from log-phase cultures of three *E. coli* strains growing at 37°C: CJ1828 (*ams-1*), CJ1827 (*rne*⁺), or CJ1827 containing plasmid pRNE101 (multicopy *rne*⁺). Equal amounts of each RNA sample (5 μg) were analyzed by ribonuclease protection using a single radiolabeled RNA probe complementary to virtually the entire *rne* transcript (nucleotides 1–3466). (A) The resulting RNase-resistant duplexes were electrophoresed on a nondenaturing 4% polyacrylamide gel. Bands corresponding to the full-length *rne* transcript (3.47-kb RNase-resistant duplex) and to a 5' segment of the *rne-lacZ* transcript *ez1* (0.91 kb) are indicated. (B) To confirm the size of the RNase-resistant duplex formed when *rne* mRNA is analyzed in this manner, a sample obtained by probing RNA from CJ1828 was also electrophoresed on a nondenaturing 0.7% agarose gel beside radiolabeled λ/*Bst*EII markers.

plasmid pRNE101. This latter observation is surprising for two reasons. First, in cells containing this plasmid, short segments of the *rne* transcript are up to 10-fold more abundant than in *rne*⁺ cells lacking the plasmid (see Fig. 3; C. Jain and J.G. Belasco, unpubl.), as expected given that the presence of pRNE101 raises the *rne* gene dosage significantly more than it reduces the half-life of the 5' and 3' segments of the *rne* transcript. Second, cells containing pRNE101 produce more RNase E protein than their plasmid-free counterparts (Fig. 4).

A model to account for these paradoxical findings is presented in detail in the Discussion. According to this model, *rne* mRNA is so long (3.6 kb) and so short-lived that few nascent *rne* transcripts survive intact until their synthesis is completed. As a consequence, full-length *rne* mRNA fails to accumulate to a detectable level in cells that overproduce RNase E. A prediction of this hypothesis is that the steady-state concentration of a truncated *rne* transcript with a reduced synthesis time should be less sensitive to RNase E overproduction. To test this hypothesis, a 2.6-kb segment was deleted in-frame from the coding region of a plasmid-borne *rne* gene, and the effect of RNase E overproduction on the concentration and half-life of the resulting truncated transcript (*rne302*) was determined. This deletion, which leaves the 5'-terminal 0.76-kb *rne* mRNA segment intact, has no effect on mRNA half-life; in *rne*⁺ cells and in cells that contain pRNE101, the 0.96-kb *rne302* message decays at essentially the same rate as the wild-type *rne* transcript (Table 3). However, in contrast to its marked effect on wild-type *rne* mRNA, RNase E overproduction reduces the steady-state concentration of the truncated *rne302* message only modestly. Whereas the abundance of full-length *rne* mRNA per *rne* gene copy decreases by more than a factor of 40 when pRNE101 is introduced into *rne*⁺ cells, the concentration of full-length *rne302* mRNA declines by only a factor of 5 (Table 3). These findings support the conclusion that the brevity of the *rne* mRNA half-life compared with its expected synthesis time makes the steady-state concentration of the wild-type message extremely sensitive to changes in its stability.

Cis-acting *rne* elements necessary for feedback regulation

To delimit further the region of the *rne* transcript necessary in *cis* for feedback regulation by RNase E, we analyzed the effect of deletions in the *rne* segment of the aforementioned *rne-lacZ* fusion (*ez1*), which comprises the promoter, 5' UTR, and first 181 codons of the *rne* gene fused in-frame to the tenth codon of *lacZ*. These constructs were introduced into an *ams-1* *E. coli* strain with low RNase E activity either by integration into the chromosome or by transformation as part of a low-copy-number plasmid. β -Galactosidase production was then assayed in the presence and absence of RNase E overproduction from plasmid pRNE101 (Table 4).

Overproduction of RNase E reduces expression of the *ez1* fusion in these cells by a factor of ~35 regardless of

Table 3. Effect of mRNA truncation on the concentration and half-life of *rne* mRNA in *rne*⁺ and pRNE101 strains

Transcript ^a	Half-life (min)		Half-life ratio ^b	mRNA concentration ratio (per gene copy) ^{b,c}
	<i>rne</i> ⁺ strain	pRNE101 strain		
<i>rne</i>	1.0	0.65 ^d	1.5	>40 ^d
<i>rne302</i>	1.1	0.8	1.4	4.7

^aHalf-life values and relative steady-state concentrations of the full-length *rne* transcript were determined in CJ1827 containing either pACYC177 (*rne*⁺ strain) or pRNE101. Corresponding values for full-length *rne302* mRNA were determined after introducing plasmid pRNE302 into the same two strains. In these assays, mRNA from exponentially growing cultures was detected by RNase protection using a radiolabeled probe complementary to virtually the entire *rne* transcript or to the entire *rne302* transcript. Data for *rne* mRNA are derived from Table 2 and Fig. 5. The *rne302* transcript is an in-frame fusion of the 5'-terminal 0.76-kb segment and 3'-terminal 0.20-kb segment of *rne* mRNA.

^bRatios represent the value in the *rne*⁺ strain divided by the value in the pRNE101 strain.

^cThe relative concentrations of the *rne* and *rne302* transcripts in the *rne*⁺ and pRNE101 strains were determined by RNase protection analysis of equal amounts of total cellular RNA and normalized to the effective *rne* or *rne302* gene copy number in each strain. The effective *rne* gene copy number is 1 in the *rne*⁺ strain and 21 in the pRNE101-containing strain (see text), whereas the corresponding values for the *rne302* gene encoded by pRNE302 (a pBR322-based plasmid) are 19 and 26, respectively. These values for the effective copy number of pRNE302 in the *rne*⁺ and pRNE101 strains were estimated by measuring the copy number of another pBR322 derivative, pEZ301, in these strains. This latter plasmid contains a copy of the *rne-lacZ* fusion *ez1*, and by comparing the level of β -galactosidase activity produced from a single-copy *ez1* fusion on the chromosome vs. a multicopy *ez1* fusion on pEZ301, we determined in each strain the effective copy number of pEZ301 and, by implication, pRNE302.

^dBecause full-length *rne* transcripts could not be detected in cells containing pRNE101, the half-life listed in this case is the average value determined for 5' and 3' segments of the transcript (see Table 2), and a lower limit is indicated for the concentration ratio of *rne* mRNA in the *rne*⁺ and pRNE101 strains.

whether the gene fusion is present on the chromosome or on a plasmid. A similar repression ratio is observed when *rne* codons 29–181 are deleted (*ez2*), demonstrating that the 5' UTR and first 28 codons of *rne* are sufficient to mediate efficient feedback repression by RNase E. This finding explains why *rne302* mRNA decays with the same kinetics as the wild-type *rne* transcript (see above), as *rne302* retains the 5' UTR and first 134 codons of *rne*. In contrast, a deletion that removes all but the last 24 nucleotides of the 361-nucleotide *rne-lacZ* 5' UTR and leaves the ribosome-binding site intact completely abolishes the responsiveness of the gene fusion to RNase E activity (*ez6*). Smaller deletions that remove internal segments (67 or 188 nucleotides) of the *ez1* 5' UTR have an intermediate effect, reducing the repres-

Table 4. Deletion mapping of cis-acting *rne* elements important for feedback regulation by RNase E

		β -Galactosidase (units) ^a		Repression ratio
		control	pRNE101	
Single-copy fusions				
<i>ez1</i>		1160	36	32
<i>ez2</i>		2460	63	39
Plasmid-borne fusions				
<i>ez1</i>		4930	134	37
<i>ez4</i>		600	112	5.4
<i>ez5</i>		2960	635	4.7
<i>ez6</i>		1370	1550	0.9
<i>ez1</i>		3660	98	37
<i>ez7</i>		2690	84	32

^aAll β -galactosidase assays were performed on extracts of an *ams-1 lacZ⁻* *E. coli* strain (CH1828) containing both an *rne-lacZ* fusion and either a multicopy *rne⁺* plasmid (pRNE101) or a control plasmid (pLG339). The various *rne-lacZ* fusions were introduced either in single copy by lysogeny of a transducing phage or in multiple copies via a pSC101-derived plasmid vector. Cells were grown at 37°C in minimal A medium. For each of the two sets of assays with plasmid-borne *rne-lacZ* fusions (*ez1*, *ez4*, *ez5*, *ez6*, and *ez1*, *ez7*), a separate measurement of β -galactosidase activity was performed with control cells that produce *ez1* mRNA to provide a same-day value for comparison of repression ratios. The 5' segment of each *rne-lacZ* hybrid transcript is represented diagrammatically. (Lines) *rne* 5' UTR; (□) 5'-terminal segment of the *rne* protein-coding region; (■) 5' portion of the *lacZ* protein-coding region; (gaps) deleted *rne* 5' UTR segment; (X) 5-nucleotide substitution (AUUUU → GGGGG) at a major cleavage site within the *rne* 5' UTR. The central and 3'-terminal segments of these transcripts are not shown.

sion ratio to 5 (*ez4*, *ez5*). These findings show that the *rne* 5' UTR is essential for RNase E-mediated down-regulation.

In *E. coli*, the *rne* and *ez1* transcripts are both cleaved within the 5' UTR at a site near the 5' end (Figs. 2 and 3). As this site lies within a 5' UTR segment that is important for feedback regulation by RNase E (Table 4), we decided to investigate whether cleavage there is a key step in this regulatory process. We first mapped the cleavage site at high resolution by using primer extension analysis to identify the 5' end of this *ez1* mRNA cleavage product and of the full-length *ez1* transcript. Cleavage was found to occur at a site 48 nucleotides downstream of the 5' terminus of the intact transcript (Fig. 6A). Interestingly, the AU-rich sequence immediately downstream of this cleavage site (AUUUU) resembles the corresponding sequence of several known RNase E cleavage sites (Ehretsmann et al. 1992) and may contribute to recognition by this ribonuclease. We then converted these five nucleotides to guanosine nucleotides (*ez7*). This mutation abolishes cleavage at the normal site but allows cleavage to occur with reduced efficiency

at new sites (Fig. 6B). Despite creating an impediment to cleavage, this mutation has little or no effect on repression of the *rne-lacZ* fusion by RNase E (Table 4) or on the steady-state concentration of the intact *rne-lacZ* hybrid transcript in *rne⁺* cells. These findings indicate that cleavage of the *rne-lacZ* transcript at this site is not essential for the degradation of this mRNA or for feedback regulation by RNase E.

The amino-terminal half of the RNase E protein is sufficient to repress rne gene expression

To identify the region of the 1061-amino-acid RNase E protein that causes repression of *rne* gene expression, carboxy-terminal deletions were created in the *rne* gene of plasmid pRNE101. The resulting deletion mutants were introduced by plasmid transformation into an *ams-1 E. coli* strain (CJ1828) containing a chromosomal *rne-lacZ* fusion (*ez1*), and the effect of each deletion on β -galactosidase production was determined in cells growing at 37°C. In addition, we assessed the ability of each RNase E mutant to complement the temperature-

Figure 6. Effect of a cleavage site mutation on the concentration of an *rne-lacZ* transcript and on its cleavage in *E. coli*. (A) Mapping of the 5' ends of an *rne-lacZ* transcript and its principal 5' UTR cleavage product. Total cellular RNA was isolated from a log-phase culture of *E. coli* strain MC1061 containing plasmid pEZ201, which encodes the *rne-lacZ* hybrid transcript *ez1*. The RNA was analyzed by primer extension with a 5'-end-labeled oligonucleotide (5'-TAACTGCCTGAAAGATCA-3') complementary to an mRNA segment located 95–112 nucleotides from the 5' end of the *rne-lacZ* transcript. The primer extension products were electrophoresed on a 4% polyacrylamide-urea gel beside a cognate sequencing ladder generated by primer extension on an *rne* DNA template. The sequencing lanes are labeled (C, U, A, G) to indicate the sequence of the RNA transcript. Bands corresponding to the 5' ends of the *rne-lacZ* transcript (5'-GUUCCGUGU...) and its principal 5'-UTR cleavage product (5'-AUUUUGCCCG...) are marked. These ends map 2–3 nucleotides upstream of the positions mapped previously for *rne* mRNA by S1 protection analysis (Claverie-Martin et al. 1991). Because high-resolution S1 mapping can be impaired by breathing at the termini of DNA/RNA duplexes, we consider the primer extension data to be more reliable. (B) Effect of a cleavage site mutation on *rne-lacZ* mRNA cleavage and abundance. Total cellular RNA was isolated from duplicate log-phase cultures of *E. coli* strain MC1061 containing plasmid pEZ201 (*ez1*) or pEZ207 (*ez7*) and growing at 37°C. Equal amounts of each RNA sample were analyzed by primer extension with the end-labeled oligonucleotide described above (5'-TAACTGCCTGAAAGATCA-3'). Bands corresponding to the 5' ends of the *rne-lacZ* transcript and its principal 5'-UTR cleavage product are marked. Together, the abundance of the multiple *ez7* 5'-UTR cleavage products is only 15–30% of the abundance of the *ez1* cleavage product, whereas the concentrations of the uncleaved *ez1* and *ez7* transcripts are the same. Because plasmid-encoded *rne-lacZ* mRNA is about nine times more abundant than chromosomally encoded *rne* mRNA in these cells, the contribution of the latter to the observed primer extension products is negligible.

sensitive growth defect caused by the *ams-1* mutation at the nonpermissive temperature (42°C).

Deletions that remove the carboxy-terminal 247, 408, or 563 amino acid residues of RNase E only partially impair its potency in feedback regulation, reducing the repression ratio from ~30 (full-length RNase E) to a factor of 6 ($\Delta 563$) to 12 ($\Delta 247$, $\Delta 408$), and all three of these mutants permit growth of the *ams-1* strain at 42°C. In contrast, deletion of 591 or 601 residues from the RNase E carboxyl terminus or of residues 29–314 near the amino terminus completely eliminates the ability of RNase E to repress *rne-lacZ* expression and to restore viability to the *ams-1* strain at the nonpermissive temperature. These findings delimit the amino-terminal RNase E domain necessary for feedback regulation and cell viability and show that these two phenomena are correlated. The data also suggest that the carboxy-terminal region of RNase E has no intrinsic ability to repress *rne* gene expression.

Discussion

Our data show that RNase E tightly regulates its own synthesis by controlling the decay rate of the *rne* transcript. Thus, a 21-fold increase in the rate of *rne* transcription results in only a 2.7-fold increase in RNase E production because of accelerated degradation of the *rne* transcript. Conversely, cells with diminished RNase E activity compensate by prolonging the lifetime of the *rne* transcript, thereby allowing as much as a fivefold increase in the rate of RNase E protein synthesis. By buffering the production of RNase E, this autoregulation mechanism helps to protect *E. coli* cells from the debilitating effects of RNase E levels that are excessively high or low.

Feedback regulation is mediated in *cis* by the 5'-terminal 0.45-kb *rne* mRNA segment, which can confer this property onto a heterologous mRNA to which it is fused. This segment comprises the 361-nucleotide *rne* 5' UTR, which is essential for feedback regulation, and the first 28 codons of the *rne* protein-coding region. Mutation of a major site of cleavage within the *rne* 5' UTR disrupts cleavage there but does not impair the ability of the 5' UTR to respond to changes in cellular RNase E activity, even though the sequence surrounding this site resembles other known RNase E cleavage sites. Presumably, disruption of this site is inconsequential either because cleavage there is unrelated to feedback regulation and does not trigger further mRNA decay or because this is only one of several potential sites where RNase E can initiate the degradation of *rne* mRNA.

Like the intact RNase E protein, the amino-terminal half of RNase E can both repress *rne* gene expression and complement the growth defect caused by a chromosomal *ams-1* mutation at 42°C. These findings suggest that the active site of this ribonuclease lies within its amino-terminal region, the downstream boundary of which maps between amino acid residues 470 and 498. Whether shorter amino-terminal RNase E fragments fail to repress *rne-lacZ* expression and complement the *ams-1*

mutation because they lack sufficient ribonuclease activity or because they are labile in *E. coli* is not clear.

Paradoxically, increasing the *rne* gene copy number in *E. coli* results in a modest increase in RNase E protein synthesis despite an unexpected reduction in the concentration of the full-length *rne* transcript. Why should the abundance of *rne* mRNA decline despite a significant increase in the copy number of the *rne* gene, and how does the cell manage to produce more RNase E from less *rne* mRNA? The likely answer is that transcription, mRNA degradation, and translation can all occur simultaneously and that a substantial fraction of *rne* messages decay cotranscriptionally. On the basis of the average rate of transcript elongation by *E. coli* RNA polymerase [30–50 nucleotides/sec (Rose et al. 1970; Jin et al. 1992)], it should take ~70–120 sec to synthesize the 3.6-kb *rne* transcript. As this synthesis time is longer than the brief half-life of *rne* mRNA in wild-type cells and in cells that overproduce RNase E, most nascent *rne* transcripts in such cells should begin to decay before their synthesis is completed. Consequently, the longevity of *rne* mRNA will affect the steady-state concentration of the full-length transcript not only by determining its rate of turnover once transcription has been completed but also by affecting the probability that nascent transcripts will remain undegraded until they reach full length. In general, for an mRNA with a half-life (τ) that is significantly shorter than its synthesis time (t) and that does not change appreciably as the nascent transcript elongates, only a small fraction of transcripts ($2^{-t/\tau}$) will survive intact until the time that their synthesis has been completed.

The substantially amplified impact of mRNA degradation under these circumstances would allow even a modest acceleration of *rne* mRNA decay to cause a marked reduction in the abundance of the full-length *rne* transcript per gene copy. In cells containing pRNE101, we calculate that this phenomenon could overwhelm the opposing effect of an increased *rne* gene dosage on the concentration of full-length *rne* mRNA if the half-life of the 3.6-kb *rne* transcript is inversely proportional to the concentration of RNase E (i.e., 22 sec in the presence of pRNE101) and if the average rate of elongation of this transcript is ~25 nucleotides/sec. In this regard, it is important to bear in mind that the measured half-lives of the 5'- and 3'-terminal *rne* mRNA segments in cells containing pRNE101 (~40 sec) only set an upper limit on the half-life of the full-length *rne* transcript, which under these conditions is too labile to be detected and which in theory might decay at a rate as high as the sum of the decay rates of its component segments. The apparent susceptibility of the nascent *rne* transcript to rapid degradation and the resulting preponderance of nascent and partially degraded *rne* transcripts also help to explain why the component segments of *rne* mRNA can be detected in cells that contain pRNE101, whereas the full-length *rne* transcript cannot (see Figs. 3 and 5).

To test the hypothesis that the sensitivity of *rne* mRNA to changes in its decay rate is in part a consequence of its relatively long synthesis time, we have ex-

amined the effect of transcript length on this phenomenon. Consistent with our model, RNase E overproduction has a much smaller effect on the cellular concentration of the truncated *rne302* message, which is just as labile as the wild-type transcript in cells containing normal and elevated levels of RNase E but only one-fourth as long. The enhanced impact of mRNA decay on the cellular concentration of transcripts whose half-life is shorter than their synthesis time may have important implications for the expression of other long transcripts that are rapidly degraded in prokaryotic organisms (e.g., transcripts of polycistronic operons) or in eukaryotic cell nuclei.

As for the ability of cells containing pRNE101 to produce two- to threefold more RNase E despite a reduced abundance of the full-length *rne* transcript, this must surely be the result of translation of nascent and partially degraded *rne* transcripts, whose synthesis is initiated more frequently in cells with an increased *rne* gene dosage. Given the potential for translating ribosomes to complete the synthesis of nascent RNase E polypeptide chains despite the degradation of *rne* mRNA segments that lie upstream, it is not surprising that the concentration of full-length *rne* transcripts is not fully representative of the available mRNA template for RNase E synthesis.

Another unexpected feature of feedback regulation by RNase E is its selective effect on gene expression. The *rne*, *ompA*, and IS10 transposase transcripts all decay more slowly when RNase E is completely inactivated at 42°C in an *rne^{ts}* *E. coli* strain (Lundberg et al. 1990; Mudd et al. 1990; Jain and Kleckner 1993); however, when these genes are fused in-frame to *lacZ*, expression of only the *rne-lacZ* fusion is sensitive to more modest changes in cellular RNase E activity. It seems unlikely that the differential response of these *lacZ* fusions can be explained by positing that the decay determinants of the parent mRNAs are absent from the *ompA-lacZ* and IS10-*lacZ* hybrid transcripts, as the *ompA-lacZ* transcript is known to share the decay characteristics and key decay determinants of *ompA* mRNA (Hansen et al. 1994). The unique behavior of the *rne-lacZ* transcript also cannot be adequately explained by the brevity of its half-life compared with its synthesis time, as some reduction in expression would be expected even for a long-lived transcript under conditions of accelerated mRNA decay. Instead, it seems more likely that the rate-determining event in the functional inactivation of the *ompA-lacZ* and IS10-*lacZ* transcripts via the RNase E degradation pathway is rather insensitive to the concentration of this enzyme. For example, mRNA degradation appears to be a multistep process (Belasco 1993), and for some mRNAs that are degraded via the RNase E pathway, cleavage by this enzyme might not be the rate-limiting step in translational inactivation except perhaps in the extreme circumstance of complete RNase E inactivation (e.g., *ams-1* cells at 42°C). Alternatively, whereas RNase E alone may be able to initiate the degradation of its own message, the efficient cleavage of other mRNAs by this ribonuclease may also require one

or more auxiliary factors, which might be present in *E. coli* at a limiting concentration. If so, the excess of RNase E under normal conditions could explain why the expression of some *lacZ* fusions is not affected by moderate changes in the cellular abundance of this protein.

Closer examination of RNase E feedback regulation should provide valuable insights into the mechanism of action of this key ribonuclease and its selective effects on gene expression. In light of evidence that the RNase E pathway for mRNA degradation involves multiple steps and additional *E. coli* proteins (Belasco 1993; He et al. 1993; Xu et al. 1993; Carpousis et al. 1994), it will be important to identify and characterize the other cellular factors involved in this regulatory pathway. The marked sensitivity of *rne-lacZ* expression to cellular RNase E activity suggests a genetic screening strategy for identifying *E. coli* gene products that influence mRNA degradation by RNase E.

Materials and methods

Bacterial strains and plasmids

The isogenic *E. coli* K-12 strains CH1826 (MC1061, *zce-726::Tn10 rne-3071*), CH1827 (MC1061, *zce-726::Tn10*), and CH1828 (MC1061, *zce-726::Tn10 ams-1*) are all derivatives of MC1061 (*araD39 Δ[ara,leu]7697, ΔlacX74 galU⁻ galK⁻ hsr⁻ hsm⁺ strA*). These strains and HAK117 (*thr leu pro argE rpsL gal ams-1 Sm^r*) have been described previously (Ono and Kuwano 1979; Mudd et al. 1990). Strains CJ1826, CJ1827, and CJ1828 were created by inserting an *rne-lacZ* fusion (*ez1*) into the chromosome of CH1826, CH1827, and CH1828, respectively (see below).

Chromosomally located *rne-lacZ* and *ompA-lacZ* gene fusions were constructed in several steps. A plasmid-borne gene fusion (*ez1*) comprising the 5' UTR and first 181 codons of the *E. coli rne* gene joined in-frame to the tenth codon of *lacZ* was constructed by inserting a 1.0-kb *BclI-HindIII* fragment from pFMK32 (Claverie-Martin et al. 1989) between the *BamHI* and *HindIII* sites of pLACZY3 (Jain 1993) to generate pEZ101. Deletion of an *EcoRV-HindIII* fragment of pEZ101 comprising *rne* codons 29–181 generated plasmid pEZ102. To construct an *ompA-lacZ* plasmid, the 5' UTR and first four codons of *ompA* were fused in-frame to *lacZ* by inserting a 650-bp *EcoRI-NruI* fragment derived from pOMPA100 (Chen et al. 1991) between the *EcoRI* and *SmaI* sites of pCJ249 (Jain 1993). These plasmid-encoded gene fusions were recombined with λ phage variant λRS45 (Simons et al. 1987) via homologous *bla* and *lac* flanking sequences. Recombinant phage, identified by their blue plaque phenotype, were then purified and allowed to lysogenize into the chromosome of various *E. coli* strains to yield single-copy gene fusions. An *E. coli* strain with a single-copy *IS10-lacZ* fusion was similarly obtained by lysogeny of λNK1167, a λRS45 derivative containing an *IS10-lacZ* fusion and obtained from Nancy Kleckner (Harvard University, Cambridge, MA).

The plasmid-borne *rne-lacZ* fusions used for deletion analysis of *cis*-acting RNA elements were first constructed in a pBR322-derived plasmid vector and then subcloned into a pSC101-derived plasmid vector. By oligonucleotide-directed mutagenesis, a unique *StuI* restriction site was created in the *rne-lacZ* 5' UTR of pEZ101 by inserting 4 bp (GGCC) at a position 24 bp upstream of the translation initiation codon. A 9.3-kb *EcoRI-StuI* fragment of the resulting plasmid was then

ligated to each of three pEZ101 fragments, all of which contained the *rne* promoter. Ligation to a 0.42-kb *EcoRI-BstUI* fragment of pEZ101 created pEZ104, which encodes *ez4*. Ligation to a 0.30-kb *EcoRI-SspI* fragment of pEZ101 created pEZ105, which encodes *ez5*. Ligation to a 0.14-kb *EcoRI-HaeIII* fragment of pEZ101 created pEZ106, which encodes *ez6*. To construct the low-copy-number *rne-lacZ* fusion plasmids pEZ201, 204, 205, and 206, a 3.6- to 4.2-kb *EcoRI-DraI* fragment containing the *rne-lacZ* fusion was excised from plasmids pEZ101, pEZ104, pEZ105, and pEZ106, respectively, and inserted between the *EcoRI* and *EcoRV* sites of pWSK30, a pSC101-based cloning vector (Wang and Kushner 1991). Plasmid pEZ207, which encodes *ez7*, was created by oligonucleotide-directed mutagenesis of pEZ201 to convert the sequence corresponding to an RNA cleavage site in the 5' UTR from ACCCATTTTGCCC to ACCGGGGGGGCC.

Plasmid pRNE101 was constructed by digesting plasmid pSC13 (Casarégola et al. 1992) with *PstI* and subcloning a 5.6-kb fragment that contains the entire *E. coli rne* gene into the *PstI* site of pACYC177 (Rose 1988a) such that transcription of the *rne* and *bla* genes was in opposite directions. The pSC101-based plasmid pFMK32, which also contains the entire *rne* gene, has been described previously (Claverie-Martin et al. 1989) and is referred to in Fig. 4 as pRNE201. Plasmid pRNE/EZ101 was constructed in multiple steps. First, a 1.1-kb *ScaI-BamHI* fragment of Bluescript KS(+) (Stratagene) was ligated to a 3.2-kb *ScaI-BamHI* fragment of pACYC177 to generate a plasmid [pACYC/BS] encoding resistance to both ampicillin and kanamycin. Then, plasmid pRNE110 was constructed by inserting a 5.6-kb *PstI* fragment of pSC13 that contains the entire *E. coli rne* gene into the *PstI* site of pACYC/BS, such that transcription of the *rne* and *bla* genes was in the same direction. Finally, a 4.2-kb *XbaI-DraI* fragment containing the *rne-lacZ* fusion *ez1* was ligated to an 8.4-kb *XbaI-ScaI* fragment of pRNE110 to generate pRNE/EZ101.

Plasmid derivatives of pRNE101 lacking portions of the *rne* protein-coding region were constructed as follows. Plasmids pRNE102, pRNE103, and pRNE104, which encode RNase E variants lacking 247, 408, or 563 carboxy-terminal amino acid residues, respectively, were created by deleting a pRNE101 fragment extending from a *BamHI* site downstream of *rne* to a *BamHI*, *MluI*, or *AflIII* site within the RNase E-coding region. Plasmids pRNE105 and pRNE106, which encode RNase E variants lacking 591 or 601 carboxy-terminal amino acid residues, respectively, were constructed by cleaving pRNE101 with *AflIII* and treating the resulting linear DNA fragment with nuclease BAL 31. The DNA was then cleaved with *BamHI*, the ends were made blunt with T4 DNA polymerase, and the DNA was circularized with incorporation of an *XbaI* linker (5'-CTAGTCTA-GACTAG-3'). The deletion endpoints of pRNE105 and pRNE106 were determined by DNA sequencing. Plasmid pRNE107 is an in-frame deletion mutant of pRNE101 lacking a 0.85-kb *EcoRV* fragment that encodes RNase E amino acid residues 29–314.

Plasmid pRNE302 was constructed in two steps. First, the 5.6-kb *PstI* fragment of pSC13 containing the entire *rne* gene was subcloned into the *PstI* site of pBR322 to give a plasmid [pRNE301] in which transcription of the *rne* and *tet* genes was in opposite directions. Deletion of a 2.6-kb *BssHII* fragment between *rne* codons 135 and 1013 then yielded pRNE302. Plasmid pEZ301, used to measure the copy number of pBR322-derived plasmids in *rne⁺* and pRNE101-containing strains, was also constructed in two steps. First, a 1.4-kb *SnaBI-NarI* fragment of pEZ101 was replaced with a polylinker sequence containing a *PstI* site and derived from pOK12 (Vieira and Messing 1991). A 5.0-kb *EcoRI-PstI* fragment containing the *rne-lacZ*

fusion was then excised from the resulting plasmid and inserted between the *EcoRI* and *PstI* sites of pBR322 to yield pEZ301.

Plasmid pLAC-RNE' was constructed by inserting the 1.0-kb *BclI*-*HindIII* *rne* fragment of pFMK32 between the *BamHI* and *HindIII* sites of plasmid Bluescript SK(+) (Stratagene). Plasmids pT7*rneFL* and pT7*rne5'* are templates for the synthesis of RNA probes complementary to *rne* mRNA. Plasmid pT7*rneFL* is a derivative of pOK12 (Vieira and Messing 1991) in which a 3.6-kb *BclI*-*BstBI* fragment of the *rne* gene and a 0.84-kb *NarI*-*BclI* fragment of pACYC184 (Rose 1988b) have been inserted between the *BglIII* and *BamHI* sites of the pOK12 polylinker such that the pACYC184 fragment is proximal to the T7 promoter. Plasmid pT7*rne5'* was constructed by inserting a 2.1-kb *PstI*-*HpaI* fragment of pRNE101 between the *PstI* and *HincII* sites of pUC21 (Vieira and Messing 1991). Plasmid pT7*rne302*, used as a template for the synthesis of an RNA probe complementary to *rne302* mRNA, was constructed by inserting a 1.7-kb *HindIII*-*DraI* fragment containing the entire *rne302* gene of pRNE302 between the *HindIII* and *SmaI* sites of plasmid Bluescript KS(+) (Stratagene). The F-based *lacI^q* plasmid pRJM386 was obtained as a gift from D. Chattoraj (National Cancer Institute, Bethesda, MD). The control plasmid pLG339 has been described previously (Stoker et al. 1982).

Ribonuclease protection assays

Radiolabeled RNA probes complementary to nucleotides 1-1257 and 1-3466 of the *rne* transcript were synthesized in vitro by transcription of pT7*rne5'* or pT7*rneFL* DNA with T7 RNA polymerase (Krupp 1988). For synthesis of the probe complementary to nucleotides 2890-3466, pT7*rneFL* DNA was linearized with *BamHI* prior to in vitro transcription. The template for preparing a radiolabeled RNA probe complementary to *rne302* mRNA was pT7*rne302* linearized with *XhoI*. The specific radioactivity of the [α -³²P]ATP used for labeling was adjusted to give incorporation, on average, of one ³²P atom per RNA molecule. Samples of total cellular RNA (5 μ g) were lyophilized with 25 nCi of probe and resuspended in 20 μ l of hybridization buffer (40 mM PIPES at pH 6.4, 1 mM EDTA, 80% formamide). The samples were heated to 90°C for 4 min and then incubated overnight at 45°C. Subsequently, 200 μ l of RNase T₁ solution (500 units) in 10 mM Tris-HCl (pH 7.5), 300 mM NaCl, and 5 mM EDTA was added, and the samples were incubated at 30°C for 1 hr. The ribonuclease digestion was stopped by addition of 225 μ l of guanidinium isothiocyanate solution (4 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.5% sodium *N*-lauroylsarcosine), tRNA (25 μ g) was added, and the RNA was precipitated with isopropanol (450 μ l). Finally, the samples were dissolved in 10 μ l of loading buffer (12.5 mM EDTA, 5% glycerol, 0.1% SDS, 0.03% bromophenol blue, 0.03% xylene cyanol) and electrophoresed on a nondenaturing 4% polyacrylamide or 0.7% agarose gel. Band intensities were quantitated using a Molecular Dynamics PhosphorImager.

Western blotting

To prepare *E. coli* protein samples for Western blotting, cells were heated to 100°C for 5 min in loading buffer (62 mM Tris-HCl at pH 6.8, 2% SDS, 10% glycerol, 0.001% bromophenol blue) and then analyzed for total protein content using the BCA assay (Stoscheck 1990). 2-Mercaptoethanol was added to a final concentration of 5%, and 5 μ g of each sample was loaded onto an SDS-polyacrylamide gel (5%) for electrophoresis. Each gel was blotted onto a Hybond-C membrane (Amersham) and probed with a polyclonal RNase E antibody directed against a protein fragment comprising RNase E amino acid residues 182-

814. RNase E-specific bands were detected using an Amersham ECL (enhanced chemiluminescence) kit. After exposing the blot to X-ray film, band intensities were quantitated using an LKB Ultrascan laser densitometer.

Other techniques

Bacterial cultures for RNA isolation, protein extraction, and β -galactosidase activity assays were grown to mid-log phase at 37°C in LB medium unless otherwise indicated. RNA isolation, primer extension reactions, and β -galactosidase assays were performed as described (Chomczynski 1993; Jain and Kleckner 1993). For mRNA half-life measurements, samples of total cellular RNA were isolated periodically from transcriptionally arrested cells, beginning 1 min after rifampicin addition (200 μ g/ml) to allow completion of the last round of transcription, and mRNA decay rates were determined by least-squares analysis of RNase protection data. Sequencing reactions were carried out using a Sequenase kit (U.S. Biochemical) according to the protocol supplied by the manufacturer. The DNA oligonucleotide used for primer extension and sequencing reactions was 5'-TAAGTGCCTGAAAGATCA-3'. Northern blotting and oligonucleotide-directed mutagenesis were performed as described (Sambrook et al. 1989; Kunkel et al. 1991).

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