MARIA CRISTINA OW
Genetic analysis of the role of ribonuclease E in RNA metabolism in *Escherichia coli* K-12.
(Under the direction of SIDNEY R. KUSHNER)

In *E. coli*, one mechanism for regulating gene expression involves the rate at which mRNAs are degraded or processed. A large body of work has shown that the principal enzyme involved in this process is ribonuclease E (RNase E). This dissertation describes research undertaken to broaden our understanding of the role of RNase E in RNA metabolism.

The carboxyl-terminus of RNase E is the scaffold for the assembly of a protein complex called the degradosome. To investigate the importance of the degradosome in RNA metabolism, we constructed truncated RNase E mutants lacking the scaffold region. In these mutants (*rneΔ225* and *rneΔ374*) mRNA decay was not affected. However, another RNase E mutant (*rneΔ610*), extensively truncated at the C-terminus, showed a dramatic increase in the longevity of mRNAs. By comparing the phenotypic properties of *rneΔ610* with the temperature-sensitive *rne-1* allele, it was possible to demonstrate that RNase E must have another essential function besides mRNA decay and rRNA processing.

The isolation of a *rneΔ610* temperature-resistant revertant, *rneΔ645*, has helped to delineate what this additional function might be. Specifically, examination of tRNA processing showed that RNase E is also required for the processing of the 5' and 3' ends of tRNA precursors. The *rneΔ645* strain processed full-length polycistronic and monocistronic tRNA transcripts 2-3-fold faster than its *rneΔ610* progenitor. These results suggest that the essential function of RNase E may be the processing of tRNA precursors.

We also examined the regulation of RNase E synthesis in the cell. The *rne* gene is transcribed from three independent promoters, p1, p2, and p3. The p1 promoter accounts for most of the *rne* transcription followed by p3 and p2. Each promoter alone was sufficient to maintain cell viability. Deletion of two of the three promoters resulted in a significant decrease of RNase E protein level. Remarkably, the large decreases in RNase E protein level were not proportionally reflected in increased mRNA decay rates. The existence of three promoters and the phenotypes associated with deleting two of the three

promoters clearly indicate that the regulation of this important ribonuclease is far more complex than previously thought.

INDEX WORDS: mRNA decay, rRNA processing, tRNA processing, *rne* promoters, Ribonuclease E, *Escherichia coli*

GENETIC ANALYSIS OF THE ROLE OF RIBONUCLEASE E IN RNA METABOLISM IN *ESCHERICHIA COLI* K-12

by

MARIA CRISTINA OW

B.S., Florida International University, 1995

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2001

GENETIC ANALYSIS OF THE ROLE OF RIBONUCLEASE E IN RNA METABOLISM IN *ESCHERICHIA COLI* K-12

by

MARIA CRISTINA OW

Approved:

Major Professor: Sidney R. Kushner

Committee: Anna Glasgow-Karls
Claiborne Glover
Richard Meagher
Michael Terns

Electronic Version Approved:

Gordhan L. Patel
Dean of the Graduate School
The University of Georgia
August 2001

DEDICATION

To my mom, dad, and brother.

ACKNOWLEDGMENTS

I am grateful to my major advisor Dr. Sidney Kushner for his support, encouragement, and guidance throughout my years in graduate school. I want to thank the members of my graduate committee, Dr. Anna Glasgow-Karls, Dr. Claiborne Glover, Dr. Richard Meagher, and Dr. Michael Terns, as well as a past member of my committee, Dr. Elliot Altman, for all their advice and constructive criticism. I also wish to thank all the past and present members of the Kushner lab for all their help.

I thank my friends in Athens for helping me keep my sanity during these past years. Also, I thank Dr. Jeff Elhai for all that he has done for me since my years at FIU to this day and for making the survival of my first grueling year of graduate school possible. Most of all, I thank my dear mom, dad, and brother for their constant love and support, for without them this work would have never been possible.

TABLE OF CONTENTS

CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

When messenger RNAs were first discovered, their defining characteristic was their instability (Brenner *et al*., 1961; Gros *et al*., 1961). It has taken several decades to appreciate that the longevity of these molecules comprises a fundamental aspect in the control of gene expression. This is because the frequency with which an mRNA is translated into its protein product is in part attributable to the length of time the mRNA stays chemically intact in the cell.

Studies in *Escherichia coli* have been instrumental in elucidating the mechanism by which this important process occurs in prokaryotes. Individual messages in *E. coli* have specified half-lives that can range from a few seconds to more than 30 minutes regardless of their length. This observation has served as an indication that the decay of an mRNA is not a random process but that it is in fact subject to tightly regulated pathways (Pedersen *et al*., 1978; Blundell *et al*., 1992; Kushner, 1996; Coburn and Mackie, 1999; Steege, 2000). The identification of numerous ribonucleases in *E. coli* has helped to define what these pathways might be. However, most of the over twenty currently characterized ribonucleases (Table 1.1) do not participate in mRNA turnover but are thought to be primarily involved in rRNA processing, tRNA processing, or act as non-specific ribonucleases (Deutscher, 1993a; 1993b). Only six ribonucleases, in fact, have been shown or have been implicated to play a role in mRNA decay (Coburn and Mackie, 1999; Steege, 2000).

Besides the characterization of ribonucleases that degrade mRNA, work during the past ten years has also identified *cis*-acting structures at both the 3' and 5' ends of mRNAs important in the regulation of mRNA turnover (Higgins *et al*., 1988; Emory *et al*., 1992). In addition, polyadenylation of mRNAs has now been shown to be an important feature of mRNA decay (Hajnsdorf *et al*., 1995; O'Hara *et al*., 1995; Sarkar, 1997). This review will focus on the current state of knowledge concerning the molecular mechanism of mRNA decay in *E. coli*.

ENDORIBONUCLEASES INVOLVED IN mRNA DECAY

These proteins are defined as ribonucleases that cleave an RNA substrate internally. Three of the six ribonucleases implicated in mRNA decay, RNase E, RNase III, and RNase G, are endoribonucleases.

A. RIBONUCLEASE E (RNase E)

Of all of the *E. coli* ribonucleases, RNase E is the principal enzyme responsible for the rate-limiting step in the decay of most messages in *E. coli* (Coburn and Mackie, 1999). It is an essential protein initially characterized as a rRNA processing enzyme required for the maturation of the 9S rRNA into a pre-5S rRNA form (Ghora and Apirion, 1978; Misra and Apirion, 1979). In a strain carrying the RNase E temperature-sensitive allele, *rne-3071*, unprocessed 9S rRNA accumulated when cells were shifted to the 43°C non-permissive temperature (Apirion and Lassar, 1978). RNase E was independently discovered as *ams-1* (for *a*ltered *m*RNA *s*tability), a thermolabile mutation that resulted in an increase in the longevity of total pulse-labeled RNA (Kuwano *et al*., 1977; Ono and Kuwano, 1979). It was not until over a decade later that work from several laboratories showed that the *rne-3071* and *ams-1* mutations were alleles of the same gene, *rne* (*ams-1* has since been renamed *rne-1*) (Mudd *et al*., 1990a; Babitzke and Kushner, 1991; Melefors and von Gabain, 1991; Taraseviciene *et al*., 1991).

Efforts to clone and sequence the *rne* gene initially led to conflicting reports regarding the size of the full-length protein (Ray and Apirion, 1980; Claverie-Martin *et al*., 1989; Chauhan *et al*., 1991; Kido *et al*., 1996). Subsequently, the complete structural gene for RNase E was inadvertently cloned as a putative structural protein that cross-reacted with a monoclonal antibody against the *Saccharomyces cerevisiae* heavy chain myosin (Casarégola *et al*., 1990; 1992; 1994). The *rne* gene is 3.6 kilobases (kb) in length and is situated at about 24 minutes (min) on the *E. coli* chromosome (Ono and Kuwano, 1980; Mudd and Higgins, 1993). Its gene product is a 1061 amino acid (aa) protein with a calculated molecular weight of 118 kDa, but it migrates aberrantly in SDS polyacrylamide

gels as 180 kDa (Casarégola *et al*., 1990; 1992; 1994). The exact catalytic mechanism of RNase E is not known, but the enzyme requires Mn^{++} or Mg^{++} and a monovalent cation such as Na^{+}, K^{+}, or NH_4^{+} for activity (Misra and Apirion, 1979).

Aside from being a 9S rRNA processing enzyme and having a role in bulk mRNA decay, RNase E has also been shown to be required for the maturation of the 5' end of the 16S rRNA (Li *et al*., 1999a; Wachi *et al*., 1999). RNase E has also been shown to be involved in the cleavage of various RNAs including RNA I (the antisense inhibitor of ColE1 plasmid replication) (Tomcsányi and Apirion, 1985a), *uncB* (a subunit of F_1F_0-ATP synthase) (Patel and Dunn, 1992; 1995), *rpsT* (ribosomal protein S20) (Mackie, 1991), *rpsO* (ribosomal protein S15) (Hajnsdorf *et al*., 1996), *dnaG* (primase) (Yajnik and Godson, 1993), and T4 bacteriophage messages (Mudd *et al*., 1990b). The processing of transcripts such as the T4 bacteriophage gene 32 (Mudd *et al*., 1988), the *E. coli pap* operon (fimbrial adhesins) (Naureckiene and Uhlin, 1996; Nilsson *et a*l., 1996), *ftsA* and *ftsZ* (cell division genes) (Cam *et al*., 1996) have also been shown to be RNase E-dependent.

Homologues or RNase E-like activities have been found in a wide array of organisms including *Rhodobacter capsulatus*, *Haemophilus influenzae*, *Streptomyces lividans* and *S. coelicolor*, *Porphyra purpurea*, *Mycobacterium tuberculosis*, *Synechocystis* sp. PCC6803, a halophilic archaeon *Haloarcula marismortui*, and humans (Claverie-Martin *et al*., 1997; Cohen, 1997; Hagège and Cohen, 1997; Kaberdin *et al*., 1998). Interestingly, the region of homology between these proteins and RNase E involves exclusively the N-terminus of the *E. coli* RNase E protein (Fig. 1.1; Kaberdin *et al*., 1998). The wide distribution of RNase E homologues throughout the various kingdoms implies that the RNase E enzyme or an RNase E-like activity has been conserved throughout evolution.

Extensive studies have been conducted to define the nature of an RNase E cleavage site. Initially, a 10 nucleotide consensus cleavage site ($ACAG^{A}/_{U}AUUUG$) was proposed based on the comparison of known RNase E cleavage sites (Tomcsányi and Apirion, 1985b). However, subsequent studies that included additional RNase E cleavage sites led to a more general recognition site. It is now believed that RNase E recognizes single-stranded

regions that lack sequence specificity but that are typically A-U rich (Coburn and Mackie, 1999). In addition, RNase E is a 5'-end dependent endoribonuclease. Evidence to support this conclusion comes from *in vitro* and *in viv*o studies using various RNase E substrates. Circularized forms of the substrates are substantially more resistant to RNase E cleavage than their linearized forms (Mackie, 1998; 2000). The hybridization of an anti-sense oligonucleotide to the 5' end also results in decreased cleavage by RNase E (Mackie, 1998). RNase E also prefers to cleave substrates with monophosphorylated 5' ends compared to triphosphorylated 5' ends (Lin-Chao and Cohen, 1991; Mackie, 1998; 2000; Tock *et al*., 2000; Spickler *et al*., 2001). Thus, these results imply that in order for RNase E to cleave an RNA substrate, it must first bind to an accessible 5' end (single-stranded and preferably monophosphorylated) prior to the initial endoribonucleolytic cleavage. These findings help to explain the reason why the decay of an mRNA generally occurs in a 5' to 3' direction as well as the "all or none" phenomenon of RNA decay (*i.e*., the general lack of visible processing intermediates in a strain) (Mackie, 2000; Spickler *et al*., 2001).

The RNase E protein apparently controls its own level in the cell via autoregulation. The first suggestion of RNase E autoregulation was put forth when it was observed that a *rne-1* mutant contained a 3-5-fold higher level of the mutant protein as compared to the level of wild-type protein in an rne^{+} strain (Claverie-Martin *et al*., 1991; Mudd and Higgins, 1993). This increased amount of Rne-1 protein was caused by stabilization of the *rne* transcript. Jain and Belasco (1995) have demonstrated that autoregulation is mediated in part by the unusually long 361 nucleotide (nt) 5' untranslated region (UTR). Based on chemical alkylation studies, the 5' UTR folds into a series of secondary structures (Diwa *et al*., 2000). Despite considerable DNA sequence divergence, the structure of the noncoding 5' leader region has been highly conserved among the *rne* mRNAs from different bacterial species (Diwa *et al*., 2000). Although the 5' UTR contains at least one RNase E cleavage site, autoregulation does not appear to be dependent on cleavage at this site (Jain and Belasco, 1995). Recently, the C-terminal region of RNase E has been implicated to be important for the autoregulation of the protein (Jiang *et al*., 2000; Ow *et al*., 2000).

The largest of the known *E. coli* ribonucleases, the RNase E protein is composed of several distinct domains (Fig. 1.1). The amino-terminal region ranging from amino acid 1 to approximately 500 contains the catalytic site as well as a putative S1 RNA binding domain (an RNA binding motif consisting of several antiparallel ß barrels originally identified in the ribosomal protein S1) (~35-125 aa) (Fig. 1.1; McDowall and Cohen, 1996; Bycroft *et al*., 1997). Mutations in the N-terminal region such as the *rne-1* (a glycine to serine replacement at residue 66) or *rne-3071* (a leucine to phenylalanine replacement at residue 68) alleles result in thermolabile RNase E activity (McDowall *et al*., 1993). RNase E polypeptides consisting of only the N-terminal domain are sufficient to maintain cell viability at 37^{o}C but not at elevated temperatures (Ow *et al*., 2000).

Following the N-terminal domain is an arginine-rich RNA-binding site (ARRBS; aa ~597-684; 29% arginines; Fig. 1.1) that has been shown to be capable of binding RNA *in vitro* (Taraseviciene *et al*., 1995; McDowall and Cohen, 1996). It contains an RNA-binding motif similar to that found in the HIV-1 Tat and Rev proteins (Burd and Dreyfuss, 1994). Flanking the ARRBS, are two proline-rich regions (aa ~526-570 and ~738-777; 34% and 18% prolines, respectively). Although the function of these regions is not known, the proline-rich regions have been implicated in the slower than expected migration of RNase E in SDS polyacrylamide gels (McDowall and Cohen, 1996). The C-terminal third of RNase E (aa ~734-1061) is dispensable for the function of the protein since polypeptides lacking this region can fully complement the *rne-1* and *rne-3071* temperature-sensitive alleles (Ray and Apirion, 1980; Claverie-Martin *et al*., 1989; Taraseviciene *et al*., 1991; Kido *et al*., 1996; Ow *et al*., 2000).

Based on various biochemical and immunological criteria, RNase E has been found to be part of a protein complex called the degradosome (Fig. 1.2). The complex has approximate molecular mass of 1.5-2.4 x 10^{6} Da and localizes near the cytoplasmic membrane (Carpousis *et al*., 1994; Liou *et al*., 2001). The other components of the degradosome include the 3' to 5' exoribonuclease polynucleotide phosphorylase (PNPase), a DEAD-box ATP-dependent RhlB RNA helicase, and the glycolytic protein enolase with

no known function in mRNA decay (Carpousis, *et al*., 1994; Py *et al*., 1994; 1996; Kido *et al*., 1996; Miczak *et al*., 1996). The C-terminal region of RNase E serves as the platform for the assembly of this complex with residues 734-738, 739-844, and 845-1045 as the binding sites of RhlB helicase, enolase, and PNPase, respectively (Fig. 1.2; Vanzo *et al*., 1998). It is also likely that the N-terminal half of RNase E is involved in dimerizing with another RNase E protein (Vanzo *et al*., 1998). The stoichiometry of the degradosome components, however, has not been determined conclusively. Other proteins reported to co-purify with RNase E include polyphosphate kinase (PPK), which catalyzes the reversible reaction of $_{n}$ATP ↔ poly (P)$_{n}$ + $_{n}$ADP, and the heat shock proteins DnaK and GroEL (Miczak *et al*., 1996; Blum *et al*., 1997).

The association of RNase E, PNPase, and RhlB in a complex suggests that these polypeptides can readily collaborate with each other to overcome physical barriers that might impede mRNA degradation. For instance, since the processivity of PNPase is generally inhibited by RNA secondary structures, RhlB could unwind the substrate so that PNPase can continue degrading it (Py *et al*., 1996). Alternatively, RNase E could cleave upstream of a stem-loop, providing a new 3' terminus for PNPase to bind to. PPK may serve to maintain the appropriate microenvironment in the degradosome by regulating the levels of ATP, poly (P), and ADP. Since ATP is required for RhlB activity and both poly (P) and ADP inhibit mRNA decay by inhibiting the degradosome and PNPase, respectively, the conversion of polyphosphate and ADP into ATP by PPK may allow the progression of the degradosome through an RNA substrate (Blum *et al*., 1997). The heat shock protein GroEL has been detected in degradosome preparations from temperature-sensitive RNase E strains but not from strains (Miczak *et al*., 1996). This is not surprising since chaperonins may be necessary to help refold a mutant RNase E protein at the nonpermissive temperature.

Protein complexes composed of ribonucleases involved in mRNA decay and/or rRNA processing have also been found in other organisms. A complex referred to as mtEXO that degrades mRNA introns has been identified in yeast mitochondria. It contains

a helicase and an exonuclease homologous to RNase II of *E. coli* (Margossian *et al*., 1996). Another complex in yeast, called the exosome, is involved in rRNA processing and mRNA degradation. It contains RNA helicases as well as several 3'-5' exonucleases related to RNase II and PNPase of *E. coli* (Mitchell *et al*., 1997; Anderson *et al*., 1998). A complex composed of a PNPase homologue and an RNase E-like protein involved in chloroplast mRNA degradation has been found in spinach (Hayes *et al*., 1996). A degradosome-like complex containing proteins homologous to RNA helicase and enolase but lacking a PNPase-like polypeptide has been detected in *Rhodobacter capsulatus* (Coburn and Mackie, 1999). It seems then, that multi-protein complexes involved in RNA processing and/or turnover are common features in prokaryotes and eukaryotes (Carpousis *et al*., 1999).

In vitro studies using purified *E. coli* degradosomes have shown that this complex is sufficient to degrade the *rpsT* transcript (Coburn and Mackie, 1998; Coburn *et al*., 1999). An *in vivo* study using an extensive RNase E truncation mutant lacking 469 or 477 amino acids from the C-terminus, removing both the degradosome scaffolding region and the ARRBS has been shown to stabilize two transcripts (López *et al*., 1999). However, as will be shown in Chapter 2, the degradosome is not necessary for normal mRNA decay in *E. coli* (Ow *et al*., 2000).

B. RIBONUCLEASE III (RNase III)

RNase III is a double-stranded specific endoribonuclease that is principally involved in the maturation of 16S and 23S rRNAs (Dunn, 1976; Nicholson, 1996). It is a ubiquitous enzyme found in both prokaryotes and eukaryotes (Court, 1993). The RNase III gene, *rnc*, is located at 58 min on the *E. coli* chromosome and is the first gene of a three gene operon, *rnc era recO* (March *et al*., 1985). The RNase III protein is 25.4 kDa and has distinct regions for catalysis, RNA binding, and dimerization with another RNase III polypeptide (Li and Nicholson, 1996).

The expression of *rnc* is also autoregulated. Specifically, RNase III removes a stabilizing element (stem-loop) from the 5' UTR of its own transcript, initiating its decay by ribonucleases other than RNase III (Matsunaga *et al*., 1996). In addition to cleaving rRNA substrates and its own message, RNase III also cleaves the *pnp* transcript, the intercistronic regions of several polycistronic mRNAs, and several bacteriophage messages (Barry *et al*., 1980; Portier *et al*., 1987; Daniels *et al*., 1988; Bardwell *et al*., 1989; Régnier and Grunberg-Manago, 1989; Faubladier *et al*., 1990).

RNase III is not an essential protein in *E. coli*, but it is required for cell viability in *Bacillus subtilis* (Babitzke *et al*., 1993; Herskovitz and Bechhofer, 2000). RNase III deletion mutants in *E. coli* accumulate 30S rRNA precursors and only show a mild phenotype (longer generation time than a strain) (Gegenheimer *et al*., 1977; Babitzke *et al*., 1993). In addition, an RNase III deletion strain does not exhibit a significant increase in the half-life of total pulse-labeled RNA. This result, together with the observation that RNase III can be deleted without deleterious effects on the cell, suggest that this endoribonuclease may not play a crucial role in mRNA degradation (Babitzke *et al*., 1993).

C. RIBONUCLEASE G (CafA / RNase G)

Extensive sequence similarity (49.5% sequence similarity and 34.1% sequence identity) exists between the N-terminus of RNase E (aa 1-498) and the protein product of another gene in *E. coli* called *cafA* (*c*ytoplasmic *a*xial *f*ilament) (McDowall *et al*., 1993; Li *et al*., 1999a; Wachi *et al*., 1999). The gene for CafA is located at 73 min on the *E. coli* chromosome, and encodes a 51 kDa protein. This locus was originally referred to as *cafA* because a strain overexpressing CafA formed chained cells due to incomplete septation. This suggested that CafA might play a role in chromosome segregation and/or cell division (Okada *et al*., 1994). Interestingly, the phenotype associated with the overexpression of CafA is reminiscent of the elongated cell phenotype observed in *rne-3071* at the nonpermissive temperature (Apirion and Lassar, 1978; Goldblum and Apirion, 1981).

Despite the sequence similarity between RNase E and CafA, CafA does not appear to be essential for cell viability (Okada *et al*., 1994). Some functional homology, however, has been implied between these two proteins from the observation that the *rne-1* mutation is partially complemented by multiple copies of the *cafA* gene and that a *cafA::cat* mutation exacerbates the temperature sensitivity of *rne-1* (Wachi *et al*., 1997).

More recently, the CafA protein was renamed RNase G (*cafA* to *rng*) because studies have shown that the CafA protein is an endoribonuclease required for the maturation of the 5' termini of the 16S rRNA (Li *et al*., 1999a; Wachi *et al*., 1999). In addition, *in vitro* studies suggest that RNase G is a 5' end-dependent endoribonuclease with a preference for 5'-monophosphorylated RNAs over 5'-triphosphorylated RNAs (Tock *et al*., 2000). A purified RNase G preparation can cleave two RNase E substrates (the 5' UTR of *ompA* and RNA I) at sites distinct from those cleaved by RNase E. RNase G, however, does not cleave the 9S rRNA (Tock *et al*., 2000).

Studies using whole genome DNA macroarray analysis as well as Northern analysis of an *rng* mutant have failed to detect any discernable differences in the steady-state level of the vast majority of transcripts in *E. coli* as well as in the decay rates of the individual mRNAs (Ow and Kushner, in preparation). This suggests that, unlike its RNase E homologue, RNase G is not likely to play a significant role in mRNA degradation.

OTHER ENDORIBONUCLEASES

A. RIBONUCLEASE P (RNase P)

This endoribonuclease is an essential enzyme responsible for the maturation of the 5' end of all tRNAs and the precursor of the 4.5S rRNA. It is composed of a protein cofactor (C5 protein) and an RNA component (M1 RNA). Although, the RNA subunit of RNase P is responsible for catalyzing the cleavage reaction, both elements are essential for *in vivo* activity. Its distribution ranges widely from archaebacteria to eukaryotes (Altman *et al*., 1995). An involvement of this ribozyme in mRNA turnover has been reported only once, in an *E. coli* study that suggested that it was involved in the maturation of the *his*

mRNA by cleaving at the base of a hairpin structure (Alifano *et al*., 1994). It has also been suggested that RNase P collaborates with RNase E in tRNA processing (Ray and Apirion, 1981a; 1981b).

B. RIBONUCLEASE I (RNase I / RNase I*)

RNase I is a nonspecific endoribonuclease. Deletion of its structural gene, *rna*, does not result in any obvious phenotype and its localization in the periplasmic space of the cell suggests that it may not contribute greatly to mRNA degradation (Meador *et al*., 1990; Zhu *et al*., 1990). RNase I* is an isoform of RNase I found in the bacterial inner membrane and has been suggested to have a role in the degradation of short oligonucleotides (Cannistraro and Kennell, 1991).

3' TO 5' EXORIBONUCLEASES INVOLVED IN mRNA DECAY

These nucleases work in a 3' to 5' direction generating mononucleotides and require a terminus to bind to for subsequent substrate cleavage. At least three exoribonucleases, PNPase, RNase II, and oligoribonuclease, play a role in mRNA degradation. Unlike in eukaryotes, no 5' to 3' exoribonucleases have been found in prokaryotes.

A. POLYNUCLEOTIDE PHOSPHORYLASE (PNPase)

PNPase catalyzes the reversible reaction of $(pN)_n + P_i \leftrightarrow (pN)_{n-1} + ppN$. The enzyme is widely distributed in both gram negative and gram positive bacteria but not in archaeabacteria or in eukaryotes (except in their organelles). Most of the early studies of *E. coli* PNPase focused on its polymerization activity (Thang *et al*., 1970). However, because intracellular inorganic phosphate levels are very high, it has been presumed that PNPase acts as one of the principal exoribonucleases in mRNA turnover (Litttauer and Soreq, 1982; Coburn and Mackie, 1999; Deutscher and Li, 2001). As a degradative enzyme, PNPase processively cleaves the 3' end of an RNA substrate phosphorolytically in the presence of inorganic phosphate, releasing nucleoside diphosphates. PNPase is one of the participants

of the phosphorolytic reactions that account for about 10% of the total exoribonucleolytic activity in *E. coli* extracts (Deutscher and Reuven, 1991). The processivity of this enzyme, however, is interrupted when it encounters RNA secondary structures. In addition, in the absence of single-stranded RNA extensions of more than 6-10 unpaired nucleotides, PNPase acts distributively instead of processively, dissociating from the substrate after each round of catalysis (Littauer and Soreq, 1982; Spickler and Mackie, 2000; Steege, 2000).

The gene for PNPase, *pnp*, maps at 71 min on the *E. coli* chromosome and is the second gene in a dicistronic operon that includes a ribosomal protein gene, *rpsO* (Portier *et al*, 1987). PNPase requires a divalent cation, with Mg^{++} as its preferred cofactor, for catalytic activity (Littauer and Soreq, 1982). The enzyme consists of three identical subunits. Each of these subunits is composed of 711 amino acids with a molecular mass of 86 kDa. The polypeptide contains two RNA-binding motifs. The first motif is a KH domain, an RNA-binding module found in many mammalian hnRNP proteins, located between residues 557 and 591. The second domain is an S1 domain at amino acids 619-691 (Bycroft *et al*., 1997). Early purifications of PNPase also contained the glycolytic enzyme enolase (Littauer and Soreq, 1982). Unknown at the time, the copurification of these two proteins was a result of their association in the degradosome (Py *et al*., 1996).

Regulation of *pnp* expression is governed at several levels. The *pnp* gene is transcribed from two promoters. The first one is located upstream of the *rpsO* gene in the *rpsO pnp* dicistronic operon and accounts for over 80% of the *pnp* transcription. The second promoter is immediately upstream of the *pnp* gene. After transcription from the first promoter, the intercistronic region of the *rpsO pnp* mRNA is subject to cleavage by RNase III. Consequently, strains lacking RNase III activity have a 10-fold increase in PNPase protein (Portier *et al*., 1987). Following RNase III cleavage, PNPase autoregulates its own translation by binding near its Shine-Dalgarno sequence (Robert-Le Meur and Portier, 1992; 1994).

It is not yet known whether the *pnp* gene is required for cell viability because existing *pnp* mutants contain a residual level of PNPase protein (less than 10% of the level

encoded by *pnp*$^{+}$; Mohanty and Kushner, unpublished observations). Mutants of *pnp*, however, show a mild growth phenotype and essentially have the same decay rate for pulse-labeled RNAs as that of a strain. Strains carrying a mutation in both the *pnp* and the *rnb* gene (encoding the exoribonuclease RNase II) are inviable, implying a functional redundancy between the two exoribonucleases (Donovan and Kushner, 1986). In addition, growth of *E. coli* at low temperatures (*e.g.*, 15^{o}C) seems to require PNPase. Evidence to support this comes from observations that the PNPase level increases by about 7-fold at 15^{o}C and that *pnp* mutant strains fail to grow at this temperature (Beran and Simons, 2001).

B. RIBONUCLEASE II (RNase II)

RNase II is encoded by the *rnb* gene located at 29 min on the *E. coli* genetic map (Zilhão *et al*., 1995a). It is a 644 aa monomeric enzyme with a molecular mass of 72.5 kDa. RNase II catalyzes the hydrolysis of an RNA substrate releasing nucleoside 5' monophosphates. Its activity is dependent on magnesium and potassium ions and is responsible for 90% of the exonucleolytic activity in *E. coli* extracts (Gupta *et al*., 1977; Deutscher and Reuven, 1991). Like PNPase, the RNase II protein also contains an S1 RNA binding domain at its C-terminus (Coburn and Mackie, 1999). Homologues of RNase II are found in evolutionarily divergent organisms from yeast to humans (Carpousis *et al*., 1999).

RNase II is highly processive but the protein easily dissociates (more so than PNPase) upon encountering stable duplex structures, leaving as many as 10 unpaired residues at the base of the stem-loop. Like PNPase, RNase II also deadenylates poly (A) tails from an RNA substrate, a characteristic of the protein that comes into play in another important feature of RNA metabolism, polyadenylation (covered later in this chapter). Because these two features are also common to PNPase, RNase II appears to paradoxically act as a repressor of exonucleolytic action by indirectly competing with PNPase (Coburn and Mackie, 1999; Mohanty and Kushner, 2000a).

RNase II has been shown to affect the stability of specific RNA substrates, such as the *rpsO* mRNA and RNA-OUT (the anti-sense RNA regulating Tn*10*/IS*10* transposition) (Pepe *et al*., 1994; Marujo *et al*., 2000). It has also been implicated in tRNA processing, specifically in the maturation of the 3' end (Li and Deutscher, 1996). Mutation of the *rnb* gene only results in a mild phenotype; however, mutation in both *rnb* and *pnp* results in inviability (Donovan and Kushner, 1996). The interrelationship between RNase II and PNPase can be observed at both the message and protein levels. For example, in PNPase-deficient cells, the expression of *rnb* is increased while in PNPase-overproducing cells, the expression of *rnb* is reduced and consequently the level of RNase II is reduced. The same holds true in *rnb* deletion strains where the PNPase level increases (Zilhão *et al*., 1995b; Zilhão *et al*., 1996). This regulatory relationship between PNPase and RNase II may explain how each of these enzymes can substitute for the other in single mutant strains, despite differences in their catalytic properties.

A recurrent theme for ribonucleases involved in mRNA degradation is their multiple levels of regulation. RNase II may be differentially regulated at the transcriptional level from two promoters with different expression levels (Zilhão *et al*., 1996). The significance of the role of these promoters in the regulation of *rnb* has not yet been detailed. Recently, a locus located downstream of *rnb* named *gmr* (*g*ene *m*odulating *R*Nase II) has been identified that appears to affect the level of RNase II at the post-translational level. Deletion of the *gmr* gene leads to a 3-fold stabilization of the RNase II protein in addition to a proportionate increase in RNase II hydrolytic activity in cell extracts. The absence of the Gmr protein also abolishes the variance in the level of RNase II in different growth media, suggesting that the regulation of RNase II level in different growth environments may be regulated by *gmr* (Cairrão *et al*., 2001). Although Gmr homologues exist in both eukaryotes and in prokaryotes, the exact function of this protein remains unknown (Cairrão *et al*., 2001).

C. OLIGORIBONUCLEASE

Of all the exoribonucleases found in *E. coli*, oligoribonuclease is the only one that has been shown to be essential for cell viability (Ghosh and Deutscher, 1999). Oligoribonuclease is a hydrolytic enzyme made up of two 20.7 kDa subunits and is encoded by the *orn* gene situated at 94 min on the *E. coli* map (Zhang *et al*., 1998). Purified oligoribonuclease requires a divalent cation (Mn^{++} or Mg^{++}) and a free 3' hydroxyl group for catalytic activity (Datta and Niyogi, 1975; Niyogi and Datta, 1975). The activity of oligoribonuclease differs from that of PNPase and RNase II in that oligoribonuclease prefers short oligoribonucleotides of 2 to 5 nucleotides in length as substrates (Yu and Deutscher, 1995). At the nonpermissive temperature, a conditional lethal mutation of the *orn* gene results in the accumulation of oligoribonucleotides of 2-5 nt in length. This means that this enzyme is solely responsible for the final step of a mRNA decay pathway, the conversion of short oligonucleotides to mononucleotides (Ghosh and Deustcher, 1999). Homologues of *orn* have been suggested to exist in eukaryotes extending from yeast to humans but are present in only certain bacteria, such as proteobacteria and mycobacteria (Deutscher and Li, 2001).

OTHER 3' TO 5' EXORIBONUCLEASES

A. RIBONUCLEASE R (RNase R)

Originally the gene encoding this enzyme, *vacB*, was shown to be involved in the establishment of virulence in *Shigella flexneri* and enteroinvasive *E. coli* (Tobe *et al*., 1992). Cloning of the *vacB* gene from *E. coli* K-12, however, revealed that it encoded an activity previously called RNase R. This enzyme was responsible for the residual hydrolytic activity in RNase II-deficient cells (Kasai *et al*., 1977; Cheng *et al*., 1998). In view of the evidence that the protein product of *vacB* is an exoribonuclease, the gene has been renamed *rnr*. *In vitro*, this enzyme acts nonspecifically on all types of RNA substrates. RNase R has a high degree (60%) of sequence similarity to RNase II. The absence of RNase R is

not detrimental to cell growth. However, the absence of both RNase R and PNPase is lethal (Cheng *et al*., 1998). Homologues of *rnr* have been found in the genomes of *Mycoplasma* to humans, but not in archaeabacteria (Cheng *et al*., 1998). Most recently, RNase R has been purified with the SsrA/ SmpB tagging system (a system that acts in clearing stalled ribosomes from mRNA) and may be involved in degrading errant mRNAs (Karzai and Sauer, 2001).

B. RIBONUCLEASE T (RNase T)

RNase T is the most effective exoribonuclease in *E. coli* for the removal of extra residues near a double-stranded RNA stem. It is this ability to approach duplexes much more closely than any other ribonuclease that makes RNase T an important enzyme in RNA metabolism. Along with RNases II, D, BN, and PH, RNase T is involved in the maturation of the 3' termini of tRNAs (Li and Deutscher, 1996). It is also essential for the maturation of the 3' end of the 5S and the 23S rRNAs as well as almost every other stable RNA (Li and Deutscher, 1995; Li *et al*., 1999b). RNase T mutant strains have the longest generation time amongst all of the other exoribonuclease mutant strains, presumably because the other exoribonucleases cannot effectively take over the functions of RNase T (Kelly and Deutscher, 1992).

C. RIBONUCLEASE PH (RNase PH)

RNase PH is the second member of the PNPase/RNase PH family. Members of this family are distinguished by their utilization of inorganic phosphate instead of H_2O as the nucleophile for RNA degradation, generating nucleoside diphosphates instead of nucleoside monophosphates. As with PNPase, RNase PH (*rph*) can act biosynthetically by adding residues to the 3' end of an RNA substrate using any nucleoside diphosphate (Ost and Deutscher, 1990). Consequently, RNase PH is homologous to PNPase, sharing motifs likely to be involved in phosphate binding and catalysis (Mian, 1997; Deutscher and Li, 2001). It is widely distributed, more so than PNPase, and is found in bacteria,

archaeabacteria, and eukaryotes. In some cases, more than one homologue is found within the same organism (Mian, 1997).

RNase PH, along with RNase T, is one of the most effective enzymes involved in the removal of extra residues following the CCA sequence in tRNA precursors. RNase PH also acts on other small stable RNAs such as M1, tm, 6S rRNA, and 4.5S rRNA (Li *et al*., 1998). Null mutants of *rph* are viable. In fact, a commonly used *E. coli* laboratory strain harbors a frameshift mutation in *rph*. The only significant phenotype associated with an *rph* mutation is a sub-optimal cellular level of pyrimidine due to the polar effect of the mutation on the downstream *pyrE* (pyrimidine biosynthesis) gene (Jensen, 1993).

D. RIBONUCLEASE D (RNase D)

RNase D (*rnd*) is involved in the maturation of the 3' end of tRNAs, but it is not one of the major participants in this process. RNase D is not an essential protein, and cells lacking RNase D do not show any growth defects (Blouin *et al*., 1983). However, RNase D becomes indispensable for cell survival in the absence of RNases II, BN, T, and PH (Kelly and Deutscher, 1992; Li and Deutscher, 1996).

E. RIBONUCLEASE BN (RNase BN)

The activity of RNase BN is highly specific for tRNA molecules, and thus it is one of the several exoribonucleases (RNases II, D, BN, T, and PH) participating in tRNA 3' end maturation. It is, however, the most inefficient of these nucleases involved in this process (Li and Deutscher, 1996). Interruption of the RNase BN gene, *rbn*, has no effect on growth because of its extremely narrow substrate specificity (Callahan and Deutscher, 1996; Deutscher and Li, 2001).

STRUCTURAL DETERMINANTS THAT AFFECT mRNA DECAY

A. 5' DETERMINANTS

Many mRNAs contain 5' structural motifs that not only confer stability on their native transcripts but can also increase the half-life of heterologous messages to which they are fused (Nierlich and Murakawa, 1996, Régnier and Arraiano, 2000). One of the best studied examples is the 5' UTR of the *ompA* message. The stabilization of *ompA* is independent of the nucleotide sequence of its 5' UTR and is principally governed by its two hairpin structures. The longevity conferred by the *ompA* 5' UTR can be transferred to a normally labile message, such as *bla*, by replacing the *bla* 5' UTR with the 5' leader sequence of *ompA* (Belasco *et al*., 1986; Emory *et al*., 1992).

RNase E has been hypothesized to be responsible for making the first endoribonucleolytic cleavage on most mRNAs (Coburn and Mackie, 1999; Steege, 2000). Consequently, features at the 5' terminus (5' phosphorylation and single-strandedness) of an RNA can directly influence the decay rate of an RNA by affecting the efficiency at which RNase E binds to the 5' end in order to initiate RNA degradation (Mackie, 1998; 2000; Spickler *et al*., 2001). For example, an mRNA without an accessible 5' end (*e.g*., not single stranded / stem-loop) would decay with a much longer half-life than one with an accessible end. Also, decay intermediates (which are 5' monophosphorylated) would be turned over with a much faster rate than the 5' triphosphorylated precursor transcript, the "all or none" phenomenon seen in RNA degradation (Mackie, 1998; 2000; Spickler *et al*., 2001).

It has also been assumed that ribosomes could interfere in the decay of a message by indirectly protecting the transcript from degradative enzymes. This concept stems from the observations of increased mRNA stability following treatment of cells with antibiotics that work by stalling ribosomes on a message (*e.g*., chloramphenicol and tetracycline). On the other hand, antibiotics (*e.g*., puromycin and kasugamycin) that promote the release of ribosomes tend to accelerate the decay rate of mRNA (Petersen, 1993). In addition, a study using a ribosome-free *lacZ* message showed that the unprotected message was more

sensitive to RNase E activity (Iost and Dreyfus, 1995). It has also been shown that the turnover of *rpsO* is sensitive to the presence of ribosomes by physically interfering with RNase E cleavage (Braun *et al*., 1998). However, the examination of translated and untranslated fragments of *bla* and *ompA* did not reveal any difference in their longevity (von Gabain *et al*., 1983). The effect of the aforementioned antibiotics is probably more complex than simple steric hindrance of a ribosome preventing ribonuclease access, since these drugs also stabilize untranslated structural RNAs. It has been suggested that inhibiting protein synthesis stimulates the synthesis of rRNA, which titrates RNases and consequently sequesters them from mRNA degradation (Grunberg-Manago, 1999). Thus, it appears that there is not a universal correlation between translation and decay and that translation can mediate the turnover of certain mRNAs and not others.

B. 3' DETERMINANTS

3' hairpins, such as Rho-independent terminators, are ubiquitous at the end of mRNAs. Another commonly found hairpin is the REP (*r*epetitive *e*xtragenic *p*alindrome) sequence. There are estimated to be over 500 REP sequences in *E. coli* and *Salmonella typhimurium*, mostly present at the ends of operons or within intercistronic regions (Higgins *et al*., 1988; Nierlich and Murakawa, 1996). A hairpin at the 3' end of an RNA can serve to reduce the processivity of exoribonucleases PNPase and RNase II. Deletion of a REP element or a Rho-independent terminator markedly destabilizes the half-life of RNAs. Conversely, the presence or the addition of these stabilizing structures can increase the longevity of RNAs (Mackie, 1987; Newbury *et al*., 1987a; 1987b; Plamann and Stauffer, 1990). Unlike in eukaryotes (Caponigro and Parker, 1996), *E. coli* mRNAs do not appear to have sequence elements or motifs at their 3' terminus that promote their rapid turnover.

POLYADENYLATION OF mRNA

mRNAs in eukaryotes undergo an extensive series of post-transcriptional modifications, including 5' capping, intron splicing, and the addition of poly (A) sequences at the 3' termini. This latter feature, the addition of adenylate residues to the 3' hydroxyl end of an mRNA using ATP as a substrate, has been extensively studied as an important parameter in the control of mRNA degradation in eukaryotes (Brawerman, 1993; Caponigro and Parker, 1996; Ross, 1996; He and Parker, 2000). Although polyadenylation in prokaryotes has not shared the same attention until recently, it was in *E. coli* that the enzyme responsible for the non-templated addition of poly (A) tails, poly (A) polymerase, was first identified many years before the discovery of polyadenylated eukaryotic mRNAs (August *et al*., 1962). Unfortunately, the erroneous notion during those early years that prokaryotic mRNAs could not be polyadenylated hindered progress in this area. Ironically, it is the poly (A) polymerase (PAP I) from *E. coli* that has always been the commercially available form of the protein. In fact, poly (A) tails have been detected in a wide array of prokaryotes, as well as in archaeabacteria and eukaryotic organelles (Sarkar, 1997).

The identification of the gene encoding PAP I and the rediscovery of poly (A) tails in *E. coli* during the early 1990's helped pave the way for the realization that polyadenylation was important for mRNA turnover in this organism (Cao and Sarkar, 1992a; 1992b). The gene for PAP I turned out to be a previously known locus, *pcnB*. This gene was identified as a mutation resulting in the reduction of the copy-number of ColE1 plasmids (Lopilato *et al*., 1986). While strains devoid of the *pcnB* gene are viable, prolonged overproduction of PAP I is lethal (Liu and Parkinson, 1989; Mohanty and Kushner, 1999a). The *E. coli* PAP I protein is a monomer of 53 kDa and has characteristics associated with members of the tRNA nucleotidyltransferase (the CCA-adding enzymes) subfamily of the nucleotidyltransferase superfamily (Yue *et al*., 1996).

Several features differ between eukaryotic and *E. coli* polyadenylation. Unlike poly (A) tails of eukaryotes (which can be hundreds of nucleotides in length), poly (A) tails in *E. coli* are relatively short, ranging between 10-40 nucleotides long (O'Hara *et al*., 1995).

Estimations of the percent of polyadenylated mRNAs in *E. coli* vary from 0.01% to less than 2% (Cao and Sarkar, 1992a; Sarkar, 1997; Mohanty and Kushner, 1999a). This is in stark contrast to the predominantly polyadenylated mRNAs in eukaryotes. In addition, unlike in eukaryotes where polyadenylation occurs downstream of an AAUAAA signal sequence, the sites of poly (A) tail addition in *E. coli* show no sequence specificity (Steege, 2000). Most importantly, polyadenylation in *E. coli* serves the opposite function to that in eukaryotes. In eukaryotes, a poly (A) tail prevents a message from being degraded (Caponigro and Parker, 1996; Ross, 1996). In *E. coli*, poly (A) tails target mRNAs for decay by providing single-stranded extensions for 3' to 5' exoribonucleolytic degradation (Marujo *et al*., 1999; Steege, 2000).

Evidence to support the destabilizing role of poly (A) tails comes from several studies. Work done to determine the mechanism by which a mutation in the *pcnB* locus affected plasmid copy number provided the first insights into the function of polyadenylation. In ColE1-type plasmids, a primer called RNA II initiates their replication. RNA II forms a hybrid with its template DNA, which is then cleaved by RNase H, producing a 3' hydroxyl terminus for the addition of dNTPs by DNA polymerase I. The antisense RNA I acts as an inhibitor of replication by hybridizing to RNA II and preventing it from initiating plasmid replication (Polisky, 1988). It was found that RNA I decay intermediates arising from RNase E-mediated cleavage were substantially stabilized (10-fold) in a *pncB* mutant. Thus, the accumulation of RNA I decay intermediates reduces plasmid copy number by sequestering RNA II. Moreover, the predominantly stabilized form of RNA I in the *pcnB* mutant lacked the poly (A) tail present in the strain (He *et al*., 1993; Xu *et al*., 1993). Studies examining individual mRNAs have given analogous results (Hajnsdorf *et al*., 1995; O'Hara *et al*., 1995). In addition, increasing the level of polyadenylation, by transiently over-expressing the PAP I protein, results in increasing the turnover rate of specific messages as well the destabilization of total pulse-labeled RNA (Mohanty and Kushner, 1999a).

It has been hypothesized that the purpose of poly (A) tails is to facilitate the degradation of an RNA substrate by providing a single-stranded platform to which 3' to 5' exoribonucleases or a degradative complex (*e.g.*, the degradosome) can bind (Kushner, 1996). Poly (A) tails have been mapped at multiple locations within a transcript (Haugel-Nielsen *et al*., 1986; Cao and Sarkar, 1992a; Xu *et al*., 1993; Goodrich and Steege, 1999). They have been found not only at the 3' end of the nascent transcript but at the 3' end of decay intermediates generated from RNase E cleavage (Haugel-Nielsen *et al*., 1986; Goodrich and Steege, 1999). Endonucleolytic cleavage by RNase E, then, appears to provide additional free 3' hydroxyl ends for polyadenylation and/or 3' to 5' exonucleolytic activity (Mohanty and Kushner, 2000a; Régnier and Arraiano, 2000; Steege, 2000).

Another gene for a potential second poly (A) polymerase, *f310*, was identified and hypothesized to account for the residual level (~10%) of polyadenylation seen in a *pcnB* deletion strain (O'Hara *et al*., 1995; Cao *et al*., 1996). Careful analysis of this gene, however, has shown that the product of the *f310* gene is not a poly (A) polymerase and hence, cannot account for the residual polyadenylation level (Mohanty and Kushner, 1999b). This residual polyadenylation, in fact, comes from an unprecedented role of PNPase acting as a poly (A) polymerase in the absence of *pcnB* (Mohanty and Kushner, 2000b).

OTHER FACTORS AFFECTING mRNA DECAY

A. MrsC

Trans-acting factors that influence mRNA degradation are not confined to ribonucleases. In fact, several non-ribonucleases have been shown to affect *E. coli* mRNA turnover rates. One of these is the MrsC protein. Mutations of the *mrsC* (for *m*RNA *s*tability) locus lead to pleiotropic effects. The *mrsC* gene was isolated in a genetic screen for temperature-sensitive alleles of *pnp*. *mrsC* mutants display longer half-lives for both total pulse-labeled RNA and for several discrete transcripts (Granger *et al*., 1998). The *mrsC* locus was found, in fact, to be allelic to the *ftsH*, *hlfB*, and *tolZ* genes (Wang *et al*.,

1998; Schumann, 1999). Previous work from independent laboratories had identified this gene as a locus involved in the high frequency of lysogeny by bacteriophage lambda (*hflB*) and in colicin sensitivity (*tolZ*) (Banuett *et al*., 1986; Qu *et al*., 1996). The *mrsC/ftsH/hlfB/tolZ* gene, in actuality, encodes a membrane-anchored ATP- and Zn^{++}-dependent metalloprotease required for the degradation of unstable proteins (Wang *et al*., 1998; Schumann, 1999), such as the lambda phage cII and cIII regulatory proteins. It has also been suggested that MrsC/FtsH/HlfB/TolZ acts as a molecular chaperone (Schumann, 1999). The mechanism by which this membrane-bound protease affects mRNA degradation has not been elucidated but it is intriguing that a recent study has suggested that RNase E and the degradosome localize to the cytoplasmic membrane (Liou *et al*., 2001), perhaps situating mRNA turnover to a specific region within the cell.

B. Hfq

Hfq (host factor I) was originally identified as a protein required for the synthesis of phage Qß RNA in *E. coli* (Franze de Fernandez *et al*., 1968). It is an abundant protein that prefers to bind single-stranded A-U-rich RNA (Senear and Steitz, 1976; Kajitani and Ishihama, 1991). The suggestion that Hfq is a post-transcriptional regulator comes from studies in which Hfq positively affects the expression of the *rpoS* (stationary phase sigma factor) at the post-transcriptional level, whereas it negatively affects the expression of *mutS* by causing a more rapid turnover of the message (Muffler *et al*., 1997; Tsui *et al*., 1997).

Additional studies have suggested that the binding of Hfq to the 5' UTR of the *ompA* message directly interferes with ribosome binding, particularly during slow growth conditions. Because the number of Hfq proteins essentially out-number ribosomes in this situation, the lack of translation may result in facilitating RNase E cleavage and commencing the rapid decay of the message (Vytvytska *et al*., 2000). In addition, it has been suggested that Hfq stimulates polyadenylation by affecting the processivity of PAP I, effectively acting akin to the poly (A) binding protein in eukaryotic polyadenylation (Hajnsdorf and Régnier, 2000).

C. CsrA / CsrB

CsrA and CsrB (*c*arbon *s*torage *r*egulatory system) are involved in controlling the expression of the *glgCAP* operon, which encodes for proteins in glycogen biosynthesis. CsrA exists as a large ribonucleoprotein complex composed of ~18 RNA-binding CsrA proteins and a CsrA-antagonizing RNA molecule called CsrB (Romeo, 1998). Mutation in the *csrA* gene leads to the stabilization of *glg* transcripts. It has been suggested that CsrA binds near the ribosome binding site of *glgC*, thereby inhibiting translation and facilitating endoribonucleolytic cleavages within the ribosome-free mRNA. The indication for CsrA acting as a *trans*-acting factor in inhibiting decay comes from the observation that the CsrB antagonizer carries 18 repeats of a Shine-Dalgarno-like sequence that would presumably titrate CsrA from the ribosome binding site (Liu *et al*., 1995; Liu and Romeo, 1997). In addition to its effect in glycogen metabolism, CsrA has been shown to affect *flhDC* (flagellum biosynthesis) expression by a mechanism similar to that seen for the *glgCAP* operon (Wei *et al*., 2001).

GENERAL MODEL OF mRNA DECAY

Because of the great diversity of mRNAs, and the numerous *cis*- and *trans*-acting factors involved in affecting their degradation, it is clear that multiple pathways for mRNA turnover must exist. Based on the information available so far, a general decay model can be formulated (Fig. 1.3). Most mRNAs studied so far decay in a 5' to 3' direction (Nierlich and Murakawa, 1996; Coburn and Mackie, 1999; Steege, 2000; Régnier and Arraiano, 2000). Since no 5' to 3' exoribonucleases have been identified in *E. coli*, the 5' to 3' directionality of decay has been attributed to the successive 5' to 3' endonucleolytic cleavages by RNase E (or RNase III for a small select group of RNAs) beginning from the 5' terminus. Initially, the 5' end-dependent RNase E (alone or associated with the degradosome) binds to the 5' triphosphorylated terminus and makes the first rate-limiting cleavage at a neighboring available site. This rate-limiting step generates 5' monophosphorylated mRNA fragments that are more efficiently processed by RNase E and

are subsequently exonucleolytically digested by 3' to 5' exoribonucleases (Xu and Cohen, 1995; Mackie, 1998; Régnier and Arraiano, 2000; Steege, 2000). Alternatively, these processing intermediates can be polyadenylated and then degraded by 3' to 5' exonucleases. The higher affinity of RNase E for processing intermediates bearing 5' monophosphorylated termini in combination with PNPase (alone or with the help of RhlB helicase in the degradosome) and/or RNase II action, in addition to multiple rounds of polyadenylation and deadenylation, assures the normally rapid disappearance of these decay intermediates (Haugel-Nielsen *et al*., 1986; Goodrich and Steege, 1999; Régnier and Arraiano, 2000; Steege, 2000). Finally, since neither PNPase nor RNase II is able to degrade small oligonucleotides, the final breakdown of RNA fragments into mononucleotides necessitates the activity of oligoribonuclease (Ghosh and Deutscher, 1999).

Although considerable advances have been made in understanding mRNA degradation in *E. coli*, many unanswered questions remain. In this dissertation, work will be presented that further examines the role of RNase E, the major player in *E. coli* mRNA decay, in RNA metabolism. One of the most exciting discoveries in the prokaryotic mRNA decay field has been the identification of a multi-protein complex called the degradosome whose members include RNase E, PNPase, and an RNA helicase. While it has been assumed that the degradosome plays an important role in mRNA turnover, the work presented in Chapter 2 shows that the assembly of this complex is not required for normal mRNA degradation. Additional results also show that the defects in mRNA decay or 9S rRNA processing associated with RNase E temperature-sensitive mutants are not the cause for their inviability at the non-permissive condition. A clue as to what might be the cause for their inviability is presented in Chapter 3. The isolation and characterization of an interesting RNase E truncation mutant led to the finding that RNase E is also involved in processing both polycistronic and monocistronic tRNA precursors. The partial rescue of tRNA processing by a temperature resistant revertant suggests that the slow rate at which tRNA precursors are processed in RNase E mutant strains may be the cause for the lethality

of these strains at high temperatures. Finally, an additional level of regulation of this important endoribonuclease is described in Chapter 4. Specifically, the *rne* gene is transcribed *in vivo* by three promoters, p1, p2, and p3. Each of these promoters can act independently of the other two and transcribe the gene with different levels of efficiency. However, transcription of the *rne* gene from only one of the three promoters results in slower growth rates, decreased RNase E levels, and defects in mRNA decay rates. In addition, RNase E autoregulation appears to be dependent on the presence of all three promoters.

REFERENCES

Alifano, P., F. Rivellini, C. Piscitelli, C. M. Arraiano, C. B. Bruni, and M. S. Carlomagno. 1994. Ribonuclease E provides substrates for ribonuclease P-dependent processing of a polycistronic mRNA. Genes Dev. 8: 3021-3031.

Altman, S., L. Kirsebom, and S. Talbot. 1995. Recent studies of RNase P. In: Söll, D., and U. RajBhandary, eds. *tRNA: Structure, Biosynthesis, and Function*. American Society for Microbiology Press, Washington, D. C. pp. 67-78.

Anderson, J. S. J., and R. Parker. 1998. The 3' to 5' degradation of yeast mRNAs is a general mechanism for mRNA turnover that requires the SKI2 DEVH box protein and 3' to 5' exonucleases of the exosome complex. EMBO J. 17: 1497-1506.

Apirion, D., and A. B. Lassar. 1978. A conditional lethal mutant of *Escherichia coli* which affects the processing of ribosomal RNA. J. Biol. Chem. 253: 1738-1742.

August, J. T., P. J. Ortiz, and J. Hurwitz. 1962. Ribonucleic acid-dependent ribonucleotide incorporation: I. Purification and properties of the enzyme. J. Biol. Chem. 237: 3786-3793.

Babitzke, P., and S. R. Kushner. 1991. The *ams* (altered mRNA stability) protein and ribonuclease E are encoded by the same structural gene of *Escherichia coli*. Proc. Natl. Acad. Sci. USA. 88: 1-5.

Babitzke, P., L. Granger, and S. R. Kushner. 1993. Analysis of mRNA decay and rRNA processing in *Escherichia coli* multiple mutants carrying a deletion in RNase III. J. Bacteriol. 175: 229-239.

Banuett, F., M. A. Hoyt, L. McFarlane, H. Echols, and I. Herskowitz. 1986. *hflB*, a new *Escherichia coli* locus regulating lysogeny and the level of bacteriophage lambda cII protein. J. Mol. Biol. 187: 213-224.

Bardwell, J. C. A., P. Régnier, S. M. Chen, Y. Nakamura, M. Grunberg-Manago, and D. L. Court. 1989. Autoregulation of RNase III operon by mRNA processing. EMBO J. 8: 3401-3407.

Barry, G., C. Squires, and C. L. Squires. 1980. Attenuation and processing of RNA from the *rplJL-rpoBC* transcription unit of *Escherichia coli*. Proc. Natl. Acad. Sci. USA 77: 3331-3335.

Belasco, J. G., G. Nilsson, A. von Gabain, and S. N. Cohen. 1986. The stability of *E. coli* gene transcripts is dependent on determinants localized to specific mRNA segments. Cell 46: 245-251.

Beran, R. K., and R. W. Simons. 2001. Cold-temperature induction of *Escherichia coli* polynucleotide phosphorylase occurs by reversal of its autoregulation. Mol. Microbiol. 39: 112-125.

Blouin, R. T., R. Zaniewski, and M. P. Deutscher. 1983. Ribonuclease D is not essential for the normal growth of *Escherichia coli* or bacteriophage T4 or the biosynthesis of a T4 suppressor tRNA. J. Biol. Chem. 258: 1423-1426.

Blum, E., B. Py, A. J. Carpousis, and C. F. Higgins. 1997. Polyphosphate kinase is a component of the *Escherichia coli* RNA degradosome. Mol. Microbiol. 26: 387-398.

Blundell, M., E. Craig, and D. Kennell. 1972. Decay rates of different mRNA in *E. coli* and models of decay. Nature New Biol. 238: 46-49.

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.

Braun, F., J. Le Derout, and P. Régnier. 1998. Ribosomes inhibit an RNase E cleavage which induces the decay of *rpsO* mRNA of *Escherichia coli*. EMBO J. 17: 4790-4797.

Brawerman, G. 1993. mRNA degradation in eukaryotic cells: an overview. In: Belasco, J. G., and G. Brawerman, eds. *Control of mRNA stability*. Academic Press, San Diego, CA. pp. 149-159.

Brenner, S., F. Jacob, and M. Meselson. 1961. An unstable intermediate carrying information from genes to ribosomes for protein synthesis. Nature 190: 576-581.

Burd, C. G., and G. Dreyfuss. 1994. Conserved structures and diversity of functions of RNA-binding proteins. Science 265: 615-621.

Bycroft, M., T. J. P. Hubbard, M. Proctor, S. M. Freud, 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.

Cairrão, F., A. Chora, R. Zilhão, A. J. Carpousis, and C. M. Arraiano. 2001. RNase II levels change according to the growth conditions: characterization of *gmr*, a new *Escherichia coli* gene involved in the modulation of RNase II. Mol. Microbiol. 39: 1550-1561.

Callahan, C., and M. P. Deutscher. 1996. Identification and characterization of the *Escherichia coli rbn* gene encoding the tRNA processing enzyme RNase BN. J. Bacteriol. 178: 7329-7332.

Cam, K., G. Rome, H. M. Krisch, and J. P. Bouché. 1996. RNase E processing of essential cell division genes mRNA in *Escherichia coli* . Nucleic Acids Res. 24: 3065-3070.

Cannistraro, V. J., and D. Kennell. 1991. RNase I*, a form of RNase I, and mRNA degradation in *Escherichia coli*. J. Bacteriol. 173: 4653-4659.

Cao, G. J., and N. Sarkar. 1992a. Poly (A) RNA in *Escherichia coli*: Nucleotide sequence at the junction of the *lpp* transcript and the polyadenylate moiety. Proc. Natl. Acad. Sci. USA 89: 7546-7550.

Cao, G. J., and N. Sarkar. 1992b. Identification of the gene for an *Escherichia coli* poly (A) polymerase. Proc. Natl. Acad. Sci. USA. 89: 10380-10384.

Cao, G. J., J. Pogliano, and N. Sarkar. 1996. Identification of the coding region for a second poly (A) polymerase in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 93: 11580-11585.

Caponigro, G., and R. Parker. 1996. Mechanisms and control of mRNA turnover in *Saccharomyces cerevisiae*. Microbiol. Rev. 60: 233-249.

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

Carpousis, A. J., N. F. Vanzo, and L. C. Raynal. 1999. mRNA degradation. A tale of poly(A) and multiprotein machines. Trends Gen. 15: 24-28.

Casarégola, S., V. Norris, M. Goldberg, and I. B. Holland. 1990. Indentification of a 180 kDa protein in *Escherichia coli* related to a yeast heavy-chain myosin. Mol. Microbiol. 4: 505-511.

Casarégola, S., A. Jacq, D. Laoudj, G. McGurk, S. Margarson, M. Tempête, V. Norris, and I. B. Holland. 1992. Cloning and analysis of the entire *Escherichia coli ams* gene: *ams* is identical to *hmp1* and encodes a 114 kDa protein that migrates as a 180 kDa protein. J. Mol. Biol. 228: 30-40.

Casarégola, S., A. Jacq, D. Laoudj, G. McGurk, S. Margarson, M. Tempête, V. Norris, and I. B. Holland. 1994. Cloning and analysis of the entire *Escherichia coli ams* gene: *ams* is identical to *hmp1* and encodes a 114 kDa protein that migrates as a 180 kDa protein. J. Mol. Biol. 238: 867.

Chauhan, A. K., A. Miczak, L. Taraseviciene, and D. Apirion. 1991. Sequencing and expression of the *rne* gene of *Escherichia coli*. Nucleic Acids Res. 19: 125-129.

Cheng, Z. F., Y. Zuo, Z., Li, K. E. Rudd, and M. P. Deutscher. 1998. The *vacB* gene required for virulence in *Shigella flexneri* and *Escherichia coli* encodes the exoribonuclease RNase R. J. Biol. Chem. 273: 14077-14080.

Claverie-Martin, F., M. R. Diaz-Torres, S. D. Yancey, and S. R. Kushner. 1989. Cloning of the altered mRNA stability (*ams*) gene of *Escherichia coli* K-12. J. Bacteriol. 171: 5479-5486.

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.

Claverie-Martin, F., M. Wang, and S. N. Cohen. 1997. *Ard-1* cDNA from human cells encodes a site-specific single-stranded endoribonuclease that functionally resembles *Escherichia coli* RNase E. J. Biol. Chem. 272: 13823-13828.

Coburn, G. A., and G. A. Mackie. 1998. Reconstitution of the degradation of the mRNA for ribosomal protein S20 with purified enzymes. J. Mol. Biol. 279: 1061-1074.

Coburn, G. A., X. Miao, D. J. Briant, and G. A. Mackie. 1999. Reconstitution of a minimal degradosome demonstrates functional coordination between a 3' exonuclease and a DEAD-box RNA helicase. Genes Dev. 13: 2594-2603.

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.

Cohen, S. N. 1997. RNase E: still a wonderfully mysterious enzyme. Mol. Microbiol. 23: 1099-1106.

Court, D. 1993. RNA processing and degradation by RNase III,. In: Belasco, J. G., and G. Brawerman, eds. *Control of mRNA stability*. Academic Press, San Diego, CA. pp. 71-116.

Daniels, D. L., M. N. Subbarao, F. R. Blattner, and H. A. Lorezon. 1988. Q-mediated late gene transcription of bacteriophage λ: RNA start point and RNase III processing sites *in vivo*. Virology 167: 568-577.

Datta, A. K., and S. K. Niyogi. 1975. Oligoribonuclease is distinct from the other known exoribonucleases of *Escherichia coli*. J. Biol. Chem. 250: 7313-7319.

Deutscher, M. P., and N. B. Reuven. 1991. Enzymatic basis for hydrolytic versus phosphorolytic mRNA degradation in *Escherichia coli* and *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA 88: 3277-3280.

Deutscher, M. P. 1993a. Ribonuclease multiplicity, diversity, and complexity. J. Biol. Chem. 268: 13011-13014.

Deutscher, M. P. 1993b. Promiscuous exoribonucleases of *Escherichia coli*. J. Bacteriol. 175: 4577-4583.

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. L. Bricker, C. Jain, and J. G. Belasco. 2000. An evolutionarily conserved RNA stem-loop functions as a sensor that directs feedback regulation of RNase E gene expression. Genes Dev.14: 1249-1260.

Donovan, W. P., and S. R. Kushner. 1986. Polynucleotide phosphorylase and ribonuclease II are required for cell viability and mRNA turnover in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA 83: 120-124.

Dunn, J. J. 1976. RNase III cleavage of single-stranded RNA. Effect of ionic strength on the fidelity of cleavage. J. Biol. Chem. 251: 3807-3814.

Emory, S. A., P. Bouvet, and J. G. Belasco. 1992. A 5' terminal stem-loop structure can stabilize mRNA in *Escherichia coli*. Genes Dev. 6: 135-148.

Faubladier, M. , K. Cam, and J. P. Bouche. 1990. *Escherichia coli* cell division inhibitor DicF-RNA of the *dicB* operon. Evidence for its generation *in vivo* by transcription termination and by RNase III-and RNase E-dependent processing. J. Mol. Biol. 21: 461-471.

Franze de Fernandez, M. T., L. Eoyang, and J. T. August. 1968. Factor fraction required for the synthesis of bacteriophage Qbeta-RNA. Nature 219: 588-590.

Gegenheimer, P., N. Watson, and D. Apirion. 1977. Multiple pathways for primary processing of ribosomal RNA in *Escherichia coli*. J. Biol. Chem. 252: 3064-3073.

Ghora, B. K., and D. Apirion. 1978. Structural analysis and *in vitro* processing to p5rRNA of a 9srRNA molecule isolated from an *rne* mutant of *E. coli*. Cell 15: 1055-1066.

Goldblum, K., and D. Apirion. 1981. Inactivation of the ribonucleic acid-processing enzyme ribonuclease E blocks cell division. J. Bacteriol. 146: 128-132.

Goodrich, A. F., and D. A. Steege. 1999. Roles of polyadenylation and nucleolytic cleavage in the filamentous phage mRNA processing and decay pathways in *Escherichia coli*. RNA 5: 972-985.

Gosh, S., and M. P. Deutscher. 1999. Oligoribonuclease is an essential component of the mRNA decay pathway. Proc. Natl. Acad. Sci. USA 96: 4372-4377.

Granger, L. L., E. B. O'Hara, R. F. Wang, F. V. Meffen, K. Armstrong, S. D. Yancey, P. Babitzke, and S. R. Kushner. 1998. The *Escherichia coli mrsC* gene is required for cell growth and mRNA decay. J. Bacteriol. 180: 1920-1928.

Gros, F., H. Hiatt, W. Gilbert, C. J. Kurland, R. W. Riseborough, and J. D. Watson. 1961. Unstable ribonucleic acid revealed by pulse-labeling of *Escherichia coli*. Nature 190: 581-585.

Grunberg-Manago, M. 1999. Messenger RNA stability and its role in control of gene expression in bacteria and phages. Annu. Rev. Genet. 33: 193-227.

Gupta, R. S., T. Kasai, and D. Schlessinger. 1977. Purification and some novel properties of RNase II. J. Mol. Chem. 252: 8945-8451.

Hagège, J. M., and S. N. Cohen. 1997. A developmentally regulated *Streptomyces* endoribonuclease resembles ribonuclease E of *Escherichia coli*. Mol. Microbiol. 25: 1077-1090.

Hajnsdorf, E., F. Braun, J. Haugel-Nielsen, and P. Régnier. 1995. Polyadenylation destabilizes the *rpsO* mRNA of *Escherichia coli*. Proc. Natl. Acad. Sci. USA 92: 3973-3977.

Hajnsdorf, E., F. Braun, J. Haugel-Nielsen, J. Le Derout, and P. Régnier. 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.

Hanjsdorf, E., and P. Régnier. 2000. Host factor Hfq of *Escherichia coli* stimulates elongation of poly (A) tails by poly (A) polymerase I. Proc. Natl. Acad. Sci. USA 97: 1501-1505.

Haugel-Nielsen, J., Hajnsdorf, E., and P. Régnier. 1996. The *rpsO* mRNA of *Escherichia coli* is polyadenylated at multiple sites resulting from endonucleolytic processing and exonucleolytic degradation. EMBO J. 15: 3144-3152.

Hayes, R., J. Kudla, G. Schuster, L. Gabay, P. Maliga, and W. Gruissem. 1996. Chloroplast mRNA 3'-end processing by a high molecular weight protein complex is regulated by nuclear encoded RNA binding proteins. EMBO J. 15: 1132-1141.

He, L., F. Söderbom, E. G. H. Wagner, U. Binnie, N. Binns, and M. Masters. 1993. PcnB is required for the rapid degradation of RNA I, the antisense RNA that controls the copy number of ColE1-related plasmids. Mol. Microbiol. 9: 1131-1142.

He, W., and R. Parker. 2000. Functions of Lsm proteins in mRNA degradation and splicing. Curr. Opin. Cell Biol. 12: 346-350.

Herskovitz, M. A., and D. H. Bechhofer. 2000. Endoribonuclease RNase III is essential in *Bacillus subtilis*. Mol. Microbiol. 38: 1027-1033.

Higgins, C. F., R. S. McLaren, and S. F. Newbury. 1988. Repetitive extragenic palindromic sequences, mRNA stability and gene expression: evolution by gene conversion? Gene 72: 3-14.

Iost, I., and M. Dreyfus. 1995. The stability of *Escherichia coli lacZ* mRNA depends upon the simultaneity of its synthesis and translation. EMBO J. 14: 3252-3261.

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.

Jensen, K. F. 1993. The *Escherichia coli* K-12 "wild types" W3110 and MG1655 have an *rph* frameshift mutation that leads to pyrimidine starvation due to low pyrE expression levels. J. Bacteriol. 175: 3401-3407.

Jiang, X., A. Diwa, A., and J. G. Belasco. 2000. Regions of RNase E important for 5'-end dependent RNA cleavage and autoregulated synthesis. J. Bacteriol. 182: 2468-2475.

Kaberdin, V. R., A. Miczak, J. S. Jakobsen, S. Lin-Chao, K. J. McDowall, and A. von Gabain. 1998. The endoribonucleolytic N-terminal half of *Escherichia coli* RNase E is evolutionarily conserved in *Synechocystis* sp. and other bacteria but not the C-terminal half, which is sufficient for degradosome assembly. Proc. Natl. Acad. Sci. USA 95: 11637-11642.

Kajitani, M., and A. Ishihama. 1991. Identification and sequence determination of the host factor gene for bacteriophage Q beta. Nucleic Acids Res. 19: 1063-1066.

Karzai, A. W., and R. T. Sauer. 2001. Protein factors associated with the SsrA•SmpB tagging and ribosome rescue complex. Proc. Natl. Acad. Sci. USA 98: 3040-3044.

Kasai T., R. S. Gupta, and D. Schlessinger. 1977. Exoribonucleases in wild type *Escherichia coli* and RNase II-deficient mutants. J. Biol. Chem. 252: 8950-8956.

Kelly, K. O., and M. P. Deutscher. 1992. The presence of only one of five exoribonucleases is sufficient to support the growth of *Escherichia coli*. J. Bacteriol. 174: 6682-6684.

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

Kushner, S. R. 1996. mRNA decay. In: Neidhardt, F. C., Curtiss III, R., Ingraham, J. L., Lin, E. C. C., Low, J., Magasanik, K. B. B., Reznikoff, W. S., Riley, M., Schaechter, M., and H.E. Umbarger, eds. *Escherichia coli and Salmonella typhimurium: Cellular and molecular biology*, vol. 2. American Society for Microbiology Press, Washington, D. C. pp. 849-860.

Kuwano, M., M. Ono, H. Endo, K. Hori, K. Nakamura, Y. Hirota, and Y. Ohnishi. 1977. Gene affecting longevity of messenger RNA: a mutant of *Escherichia coli* with altered mRNA stability. Mol. Gen. Genet. 154: 279-285.

Li, Z., and M. P. Deutscher. 1995. The tRNA processing enzyme RNase T is essential for maturation of 5S rRNA. Proc. Natl. Acad. Sci. USA 92: 6883-6886.

Li, Z., and M. P. Deutscher. 1996. Maturation pathways for *E. coli* tRNA precursors: a random multienzyme process *in vivo*. Cell 86: 503-512.

Li, H., and A. W. Nicholson. 1996. Defining the enzyme binding domain of a ribonucleaes III processing signal. Ethylation interference and hydroxyl radical footprinting using catalytically inactive RNase III mutants. EMBO J. 15: 1421-1433.

Li, Z., S. Pandit, and M. P. Deutscher. 1998. 3' exoribonucleolytic trimming is a common feature of the maturation of small, stable RNAs in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 95: 2856-2861.

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

Li, Z., S. Pandit, and M. P. Deutscher. 1999b. Maturation of the 23S ribosomal RNA requires the exoribonuclease RNase T. RNA 5: 139-146.

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

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

Littauer, U. Z., and H. Soreq. 1982. Polynucletide phosphorylase, In: P. D. Boyer, ed. *The enzymes*, vol. XV. Academic Press, New York. pp. 517-553.

Liu, M. Y., H. Yang, and T. Romeo. 1995. The product of the pleiotropic *Escherichia coli* gene *csrA* modulates glycogen biosynthesis via effects on mRNA stability. J. Bacteriol. 177: 2663-2672.

Liu, M. Y., and T. Romeo. 1997. The global regulator CsrA of *Escherichia coli* is a specific mRNA-binding protein. J. Bacteriol. 179: 4639-4642.

Liu, J., and J. S. Parkinson. 1989. Genetics and sequence analysis of the *pcnB* locus, an *Escherichia coli* gene involved in plasmid copy number control. J. Bacteriol. 171: 1254-1261.

López, P. J., I. Marchand, S. A. Joyce, and M. Dreyfus. 1999. The C-terminal half of RNase E, which organizes the *Escherichia coli* degradosome, participates in mRNA degradation but not rRNA processing *in vivo*. Mol. Microbiol. 33: 188-189.

Lopilato, J., S. Bortner, and J. Beckwith. 1986. Mutations in a new chromosomal gene of *Escherichia coli* K-12, *pcnB*, reduce plasmid copy number of pBR322 and its derivatives. Mol. Gen. Genet. 205: 285-290.

Mackie, G. A. 1987. Posttranscriptional regulation of ribosomal protein S20 and stability of the S20 mRNA species. J. Bacteriol. 169: 2697-701.

Mackie, G. A. 1991. Specific endonucleolytic cleavage of the mRNA for ribosomal protein S20 of *Escherichia coli* requires the product of the *ams* gene *in vivo* and *in vitro*. J. Bacteriol. 173: 2488-2497.

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

Mackie, G. A. 2000. Stabilization of circular *rpsT* mRNA demonstrates the 5'-end dependence of RNase E action *in vivo*. J. Biol. Chem. 275: 25069-25072.

March, P. E., J. Ahnn, and M. Inouye. 1985. The DNA sequence of the gene (*rnc*) encoding ribonuclease III of *Escherichia coli*. Nucleic Acids Res. 13: 4677-4685.

Margossian, S. P., H. Li, H. P. Zassenhaus, and R. A. Butow. 1996. The DExH box protein Suv3p is a component of a yeast mitochondrial 3' to 5' exoribonuclease that suppresses group I intron toxicity. Cell 84: 199-209.

Marujo, P. E., E. Hajnsdorf, J. Le Derout, R. Andrade, C. M. Arraiano, and P. Régnier. 2000. RNase II removes the oligo(A) tails that destabilize the *rpsO* mRNA of *Escherichia coli*. RNA 6:1185-1193.

Matsunaga, J., E. L. Simons, and R. W. Simons. 1996. RNase III autoregulation: structure and function of *rncO*, the posttranscriptional "operator." RNA 2: 1228-1240.

McDowall, K. J., R. G. Hernandez, S. Lin-Chao, and S. N. Cohen. 1993. The *ams-1* and *rne-3071* temperature sensitive mutations in the *ams* gene are in close proximity to each other and cause substitutions within a domain that resembles a product of the *Escherichia coli mre* locus. J. Bacteriol. 175: 4245-4249.

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.

Meador, J., B. Cannon, V. J. Cannistraro, and D. Kennell. 1990. Purification and characterization of *Escherichia coli* RNase I. Comparisons with RNase M. Eur. J. Biochem. 187: 549-553.

Melefors, Ö., and A. von Gabain. 1991. Genetic studies of cleavage-initiated mRNA decay and processing of ribosomal 9s rRNA show that the *Escherichia coli ams* and *rne* loci are the same. Mol. Microbiol. 5: 857-864.

Mian, I. S. 1997. Comparative sequence analysis of ribonucleases HII, III, II, PH, and D. Nucleic Acids Res. 25: 3187-3195.

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.

Misra, T. K., and D. Apirion. 1979. RNase E, an RNA processing enzyme from *Escherichia coli*. J. Biol. Chem. 254: 11154-11159.

Mitchell, P., E. Petfalski, A. Sheuchenko, M. Mann, and D. Tollervey. 1997. The exosome: a conserved eukaryotic RNA processing complex containing multiple 3'-5' exoribonucleases. Cell 91: 457-466.

Mohanty, B. K., and S. R. Kushner. 1999a. Analysis of the function of *Escherichia coli* poly (A) polymerase I in RNA metabolism. Mol. Microbiol. 34: 1094-1108.

Mohanty, B. K., and S. R. Kushner. 1999b. Residual polyadenylation of poly (A) polymerase I (*pcnB*) mutants of *Escherichia coli* does not result from activity encoded by the *f310* gene. Mol. Microbiol. 34: 1109-1119.

Mohanty, B. K., and S. R. Kushner. 2000a. Polynucleotide phosphorylase, RNase II and RNase E play different roles in the *in vivo* modulation of polyadenylation in *Escherichia coli*. Mol. Microbiol. 36: 982-994.

Mohanty, B. K., and S. R. Kushner. 2000b. Polynucleotide phosphorylase functions both as a 3' ' 5' exonuclease and a poly (A) polymerase in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 97: 11966-11971.

Mudd, E. A., P. Prentki, D. Belin, and H. M. Krisch. 1988. Processing of unstable bacteriophage T4 gene 32 mRNAs into a stable species requires *Escherichia coli* ribonuclease E. EMBO J. 7: 3601-3607.

Mudd, E. A., H. M. Krisch, and C. F. Higgins. 1990a. RNaseE, an endoribonuclease, has a general role in the chemical decay of *Escherichia coli* mRNA: evidence that *rne* and *ams* are the same genetic locus. Mol. Microbiol. 4: 2127-2135.

Mudd, E. A., A. J. Carpousis, and H. M. Krisch. 1990b. *Escherichia coli* RNase E has a role in the decay of bacteriophage T4 mRNA. Genes Dev. 4: 873-881.

Mudd, E. A., and C. F. Higgins. 1993. *Escherichia coli* endoribonuclease RNase E: autoregulation of expression and site specific cleavage of mRNA. Mol. Microbiol. 9: 557-568.

Muffler, A., D. D. Traulsen, D. Fischer, and R. Hengge-Aronis. 1997. The RNA-binding protein HF-1 plays a global regulatory role which is largely, but not exclusively, due to its role in expression of the sigmaS subunit of RNA polymerase in *Escherichia coli*. J. Bacteriol. 179: 297-300.

Naureckiene, S., and B. E. Uhlin. 1996. *In vitro* analysis of mRNA processing by RNase E in the *pap* operon of *Escherichia coli*. Mol. Microbiol. 21: 55-68.

Newbury, S. F., N. H. Smith, and C. F. Higgins. 1987a. Differential mRNA stability controls relative gene expression within a polycistronic operon. Cell 51: 1131-43.

Newbury, S. F., N. H. Smith, E. C. Robinson, I. D. Hiles, and C. F. Higgins. 1987b. Stabilization of translationally active mRNA by prokaryotic REP sequences. Cell 48: 297-310.

Nicholson, A. W. 1996. Structure, reactivity, and biology of double-stranded RNA. Prog. Nucleic Acid Res. Mol. Biol. 52: 1-65.

Nierlich, D. P., and G. J. Murakawa. 1996. The decay of bacterial messenger RNA. Prog. Nucleic Acids Res. Mol. Biol. 52: 153-216.

Nigoyi, S. K., and A. K. Datta. 1975. A novel oligoribonuclease of *Escherichia coli*. I. Isolation and properties. J. Biol. Chem. 250: 7307-7312.

Nilsson, P., S. Naureckiene, and B. E. Uhlin. 1996. Mutations affecting mRNA processing and fimbrial biogenesis in the *Escherichia coli pap* operon. J. Bacteriol. 178: 683-690.

O'Hara, E. B., J. A. Chekanova, C. A. Ingle, Z. R. Kushner, E. Peters, and S. R. Kushner. 1995. Polyadenylation helps regulate mRNA decay in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 92: 1807-1811.

Okada, Y., M. Wachi, A. Hirata, K. Susuki, K. Nagai, and M. Matsuhashi. 1994. Cytoplasmic axial filaments in *Escherichia coli* cells: possible function in the mechanism of chromosome segregation and cell division. J. Bacteriol. 176: 917-922.

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

Ono, M., and K. Kuwano. 1980. Chromosomal location of a gene for chemical longevity of messenger ribonucleic acid in a temperature sensitive mutant of *Escherichia coli*. J. Bacteriol. 142: 325-326.

Ost, K. A., and M. P. Deutscher. 1990. RNase PH catalyzes a synthetic reaction, the addition of nucleotides to the 3' end of RNA. Biochimie 72: 813-818.

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.

Patel, A. M., and S. D. Dunn. 1992. RNase E-dependent cleavages in the 5' and 3' regions of the *Escherichia coli unc* mRNA. J. Bacteriol. 174: 3541-3548.

Patel, A. M., and S. D. Dunn. 1995. Degradation of *Escherichia coli uncB* mRNA by multiple endonucleolytic cleavages. J. Bacteriol. 177: 3917-3922.

Pedersen, S., S. Reech, and J. D. Friesen. 1978. Functional mRNA half-lives in *E. coli*. Mol. Gen. Genet. 166: 329-336.

Pepe, C. M., S. Maslesa-Galic, and R. W. Simons. 1994. Decay of the IS*10* antisense RNA by 3' exoribonucleases: evidence that RNase II stabilizes RNA-OUT against PNPase attack. Mol. Microbiol. 13: 1133-1142.

Petersen, C. 1993. Translation and mRNA stability in bacteria: a complex relationship. In: Belasco, J. G., and G. Brawerman, eds. *Control of mRNA stability*. Academic Press, San Diego, CA. pp. 117-145.

Plamann, M. D., and G. V. Stauffer. 1990. *Escherichia coli glyA* mRNA decay: the role of 3' secondary structure and the effects of the *pnp* and *rnb* mutations. Mol. Gen. Genet. 220: 301-6.

Polisky, B. 1988. ColE1 replication control circuitry: sense from antisense. Cell 55: 929-932.

Portier, C., L. Dondon, M. Grunberg-Manago, and P. Régnier. 1987. The first step in the functional inactivation of the *Escherichia coli* polynucleotide phosphorylase messenger is a ribonuclease III processing at the 5' end. EMBO J. 6: 2165-2170.

Py, B., H. Cauton, E. A. Mudd, and C. F. Higgins. 1994. A protein complex mediating mRNA degradation in *Escherichia coli*. Mol. Microbiol. 14: 717-729.

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.

Qu, J. N., S. I. Makino, H. Adachi, Y. Koyama, Y. Akiyama, K. Ito, T. Tomoyasu, T. Ogura, and H. Matsuzawa. 1996. The *tolZ* gene of *Escherichia coli* is identified as the *ftsH* gene. J. Bacteriol. 178: 3457-3461.

Ray, A., and D. Apirion. 1980. Cloning the gene for ribonuclease E, an RNA processing enzyme. Gene 12: 87-94.

Ray, B. K., and D. Apirion. 1981a. Transfer RNA precursors are accumulated in *Escherichia coli* in the absence of RNase E. *Eur J Biochem* 114: 517-524.

Ray, B. K.,and D. Apirion. 1981b. RNAase P is dependent on RNAase E action in processing monomeric RNA precursors that accumulate in an RNAase E-mutant of *Escherichia coli*. J. Mol. Biol. 149: 599-617.

Régnier, P., and M. Grunberg-Manago. 1989. Cleavage by RNase III in the transcripts of the *metY-nusA-infB* operon of *Escherichia coli* releases the tRNA and initiates the decay of the downstream mRNA. J. Mol. Biol. 210: 293-302.

Régnier, P., and C. M. Arraiano. 2000. Degradation of mRNA in bacteria: emergence of ubiquitous features. Bioessays 22: 235-244.

Robert-Le Meur, M., and C. Portier. 1992. *E. coli* polynucleotide phosphorylase expression is autoregulated through and RNase III-dependent mechanism. EMBO J. 11: 2633-2641.

Robert-Le Meur, M., and C. Portier. 1994. Polynucleotide phosphorylase of *Escherichia coli* induces the degradation of its RNase III processed messenger by preventing its translation. Nucleic Acids Res. 22: 397-403.

Romeo, T. 1998. Global regulation by the small RNA-binding protein CsrA and the non-coding RNA molecule CsrB. Mol. Microbiol. 29: 1321-1330.

Ross, J. 1996. Control of messenger RNA stability in higher eukaryotes. Trends Genet. 12: 171-175.

Sarkar, N. 1997. Polyadenylation of mRNA in prokaryotes. Annu. Rev. Biochem. 66: 173-197.

Schumann, W. 1999. FtsH--a single-chain charonin? FEMS Microbiol. Rev. 23: 1-11.

Senear, A. W., and J. A. Steitz. 1976. Site-specific interaction of Qbeta host factor and ribosomal protein S1 with Qbeta and R17 bacteriophage RNAs. J. Biol. Chem. 251: 1902-1912.

Spickler, C., and Mackie, G. A. 2000. Action of RNase II and polynucleotide phosphorylase against RNAs containing stem-loops of defined structure. J. Bacteriol. 182: 2422-2427.

Spickler, C., V. Stronge, and G. A. Mackie. 2001. Preferential cleavage of degradative intermediates of *rpsT* mRNA by the *Escherichia coli* RNA degradosome. J. Bacteriol. 183: 1106-1109.

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

Taraseviciene, L., A. Miczak, and D. Apirion. 1991. The gene specifying RNase E (*rne*) and a gene affecting mRNA stability (*ams*) are the same gene. Mol. Microbiol. 5: 851-855.

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

Thang, M. N., R. A. Harvey, and M. Grunberg-Manago. 1970. Model for the elongation of polynucleotide chains by polynucleotide phosphorylase. J. Mol. Biol. 53: 261-80.

Tobe, T., C. Sasakawa, N. Okada, Y. Honma, and M. Yoshikawa. 1992. *vacB*, a novel chromosomal gene required for expression of virulence genes on the large plasmid of *Shigella flexneri*. J. Bacteriol. 174: 6359-6367.

Tock, M. R., A. P. Walsh, G. Carroll, and K. J. McDowall. 2000. The CafA protein required for the 5'-maturation of 16S rRNA is a 5'-end-dependent ribonuclease that has context-dependent broad sequence specificity. J. Biol. Chem. 275: 8726-8732.

Tomcsányi, T., and D. Apirion. 1985a. Processing enzyme ribonuclease E specifically cleaves RNA I, an inhibitor of primer formation in plasmid DNA synthesis. J. Mol. Biol. 185: 713-720.

Tomcsányi, T., and D. Apirion. 1985b. Processing enzyme ribonuclease E specifically cleaves RNA I, an inhibitor of primer formation in plasmid DNA synthesis. J. Mol. Biol. 185: 713-720.

Tsui, H. C., G. Feng, and M. E. Winkler. 1997. Negative regulation of *mutS* and *mutH* repair gene expression by the Hfq and RpoS global regulators of *Escherichia coli* K-12. J. Bacteriol. 179: 7476-7487.

Vanzo, N. F., Y. S. Li, B. Py, E. Blum, C. F. Higgins, L. C. Raynal, H. M. Krisch, and A. J. Carpousis. 1998. Ribonuclease E organizes the protein interactions in the *Escherichia coli* RNA degradosome. Genes Dev. 12: 2770-2781.

Vytvytska, O., I. Moll, V. R. Kaberdin, A. von Gabain, and U. Bläsi. 2000. Hfq (HF1) stimulates *ompA* mRNA decay by interfering with ribosome binding. Genes Dev. 14: 1109-1118.

von Gabain, A., J. G. Belasco, J. L. Schottel, A. C. Y. Chang, and S. N. Cohen. 1983. Decay of mRNA in *Escherichia coli*: investigation of the fate of specific segments of transcripts. Proc. Natl. Acad. Sci. USA 80: 653-657.

Wachi, M., G. Umitsuki, and K. Nagai. 1997. Functional relationship between *Escherichia coli* RNase E and the CafA protein. Mol. Gen. Genet. 253: 515-519.

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

Wang, R. F., E. B. O'Hara, M. Aldea, C. I. Bargmann, H. Gromley, and S. R. Kushner. 1998. *Escherichia coli mrsC* is an allele of *hflB*, encoding a membrane-associated ATPase and protease that is required for mRNA decay. J. Bacteriol. 180: 1929-1938.

Wei, B. L., A. M. Brun-Zinkernagel, J. W. Simecka, B. M. Prüß, P. Babitzke, and T. Romeo. 2001. Positive regulation of motility and *flhDC* expression by the RNA-binding protein CsrA of *Escherichia coli*. Mol. Microbiol. 40: 245-256.

Xu, F., S. Lin-Chao, and S. N. Cohen. 1993. The *Escherichia coli pcnB* gene promotes adenylation of antisense RNA I of ColE1-type plasmids *in vivo* and degradation of RNA I decay intermediates. Proc. Natl. Acad. Sci. USA 90: 6756-6760.

Xu, F., and S. N. Cohen. 1995. RNA degradation in *Escherichia coli* regulated by 3' adenylation and 5' phosphorylation. Nature 374: 180-183.

Yajnik, V., and G. Nigel Godson. 1993. Selective decay of *Escherichia coli dnaG* messenger RNA is initiated by RNase E. J. Biol. Chem. 268: 13253-13260.

Yu, D., and M. P. Deutscher. 1995. Oligoribonuclease is distinct from the other known exoribonucleases of *Escherichia coli*. J. Bacteriol. 177: 4137-4139.

Yue, D., N. Maizels, and A. M. Weiner. 1996. CCA-adding enzymes and poly (A) polymerase are all members of the same nucloetidyl transferase superfamily: Characterization of the CCA-adding enzyme from the archaeal hyperthermophile *Sulfolobus shibata*. RNA 2: 895-908.

Zhang, X., L. Zhu, and M. P. Deutscher. 1998. Oligoribonuclease is encoded by a highly conserved gene in the 3'-5' exonuclease superfamily. J. Bacteriol. 180: 2779-2781.

Zhu, L., T. Gangopadhyay, K. P. Padmanabha, and M. P. Deutscher. 1990. *Escherichia coli rna* gene encoding RNase I: cloning, overexpression, subcellular distribution of the enzyme, and use of an *rna* deletion to identify additional RNases. J. Bacteriol. 172: 3146-3151.

Zilhão, R., F., Cairrao, P. Régnier, and C. M. Arraiano. 1995a. Precise physical mapping of the *Escherichia coli rnb* gene, encoding ribonuclease II. Mol. Gen. Genet. 248: 242-246.

Zilhão, R., P. Régnier, and C. M. Arraiano. 1995b. The role of endonucleases in the expression of ribonuclease II in *Escherichia coli*. FEMS Microbiol. Lett. 130: 237-244.

Zilhão, R., F., Cairrao, P. Régnier, and C. M. Arraiano. 1996. PNPase modulates RNase II expression in *Escherichia coli*: implications for mRNA decay and cell metabolism. Mol. Microbiol. 20: 1033-1042.

Table 1.1. Selected ribonucleases of *E. coli*.

Enzyme	Gene	Map position (min)	Subunit MW (kDa)	Substrate(s)	Major characteristic(s)	Essential for viability
RNase E	*rne*	24	118	mRNAs, 9S and 16S rRNA, tRNAs	endoribonuclease, 5' P >> 5' PPP, single-stranded specific	Yes
RNase III	*rnc*	58	25	16S rRNA, 23S rRNA, some mRNAs	endoribonuclease, double-stranded specific	No
RNase G	*rng*	73	51	16S rRNA, some mRNAs?	endoribonuclease, 5' P >> 5' PPP	No
RNase P	*rnpA*[c] *rnpB*[d]	84 70	14 376 nt	tRNAs, 4.5S rRNA	endoribonuclease	Yes
RNase I	*rna*	14	27	non-specific	endoribonuclease	No

PNPase	*pnp*	71	86	mRNAs	3' to 5' exoribonuclease (phosphorolytic), also biosynthetic	ND[a, b]
RNase II	*rnb*	29	73	mRNAs, tRNAs	3' to 5' exoribonuclease (hydrolytic)	No[b]
Oligoribonuclease	*orn*	94	21	oligoribonucleotides	3' to 5' exoribonuclease (hydrolytic)	Yes
RNase R	*rnr*	95	92	non-specific	3' to 5' exoribonuclease (hydrolytic)	No
RNase T	*rnt*	37	23	tRNAs	3' to 5' exoribonuclease (hydrolytic)	No
RNase PH	*rph*	82	26	tRNAs	3' to 5' exoribonuclease (phosphorolytic)	No
RNase D	*rnd*	40	43	tRNAs	3' to 5' exoribonuclease (hydrolytic)	No

RNase BN	*rbn*	88	33	tRNAs	3' to 5' exoribonuclease (hydrolytic)	No

[a] Not yet determined.

[b] PNPase and RNase II double mutants are inviable.

[c] *rnpA* encodes for the protein subunit (C5 protein) of RNase P.

[d] *rnpB* encodes for the catalytic RNA subunit (M1 RNA) of RNase P.

Figure 1.1. Schematic of the RNase E protein. The 1061 amino acid long RNase E protein consists primarily of three regions. The N-terminus (amino acid 1 to 498) is the catalytic region containing a putative S1 RNA binding domain (aa ~35-125). The catalytic region is followed by an arginine-rich RNA binding site (ARRBS) from residues 597 to 684. Finally, the degradosome scaffolding region maps to the C-terminus of the protein from residues 734 to 1045.

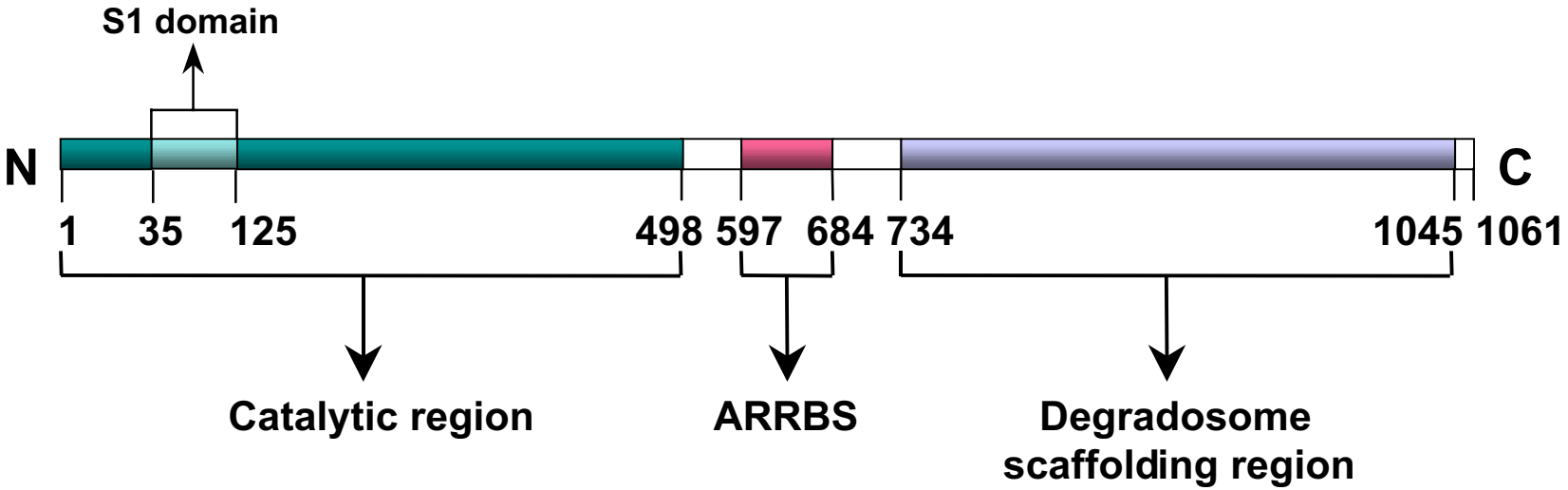

S1 domain
N
C
1
35
125
498
597
684
734
1045
1061
Catalytic region
ARRBS
Degradosome
scaffolding region

Figure 1.2. The RNase E-based degradosome. The complex consists of RNase E, the 3' to 5' exoribonuclease PNPase, the RNA helicase RhlB, and enolase. The C-terminus of RNase E serves as the platform for the formation of the degradosome with residues 734-738, 739-844, and 845-1045 involved in the binding of RhlB, enolase, and PNPase, respectively. The exact stoichiometry of the protein components has not been determined.

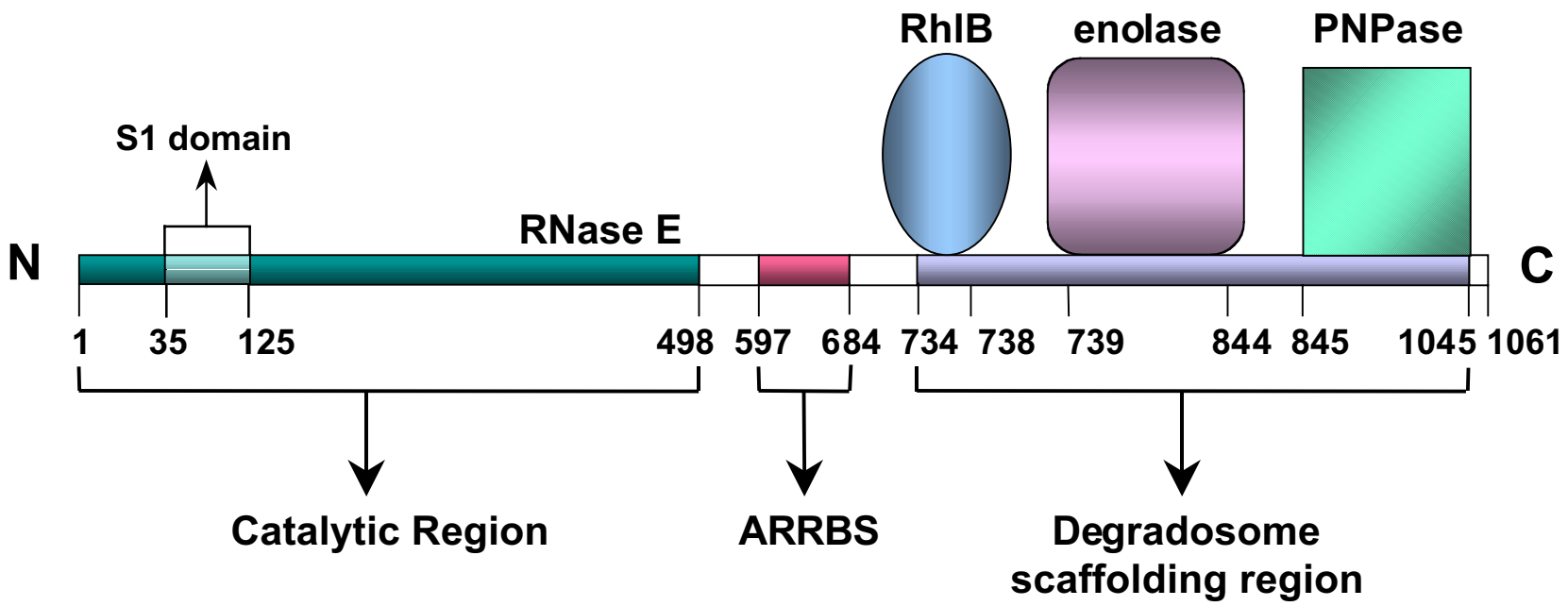
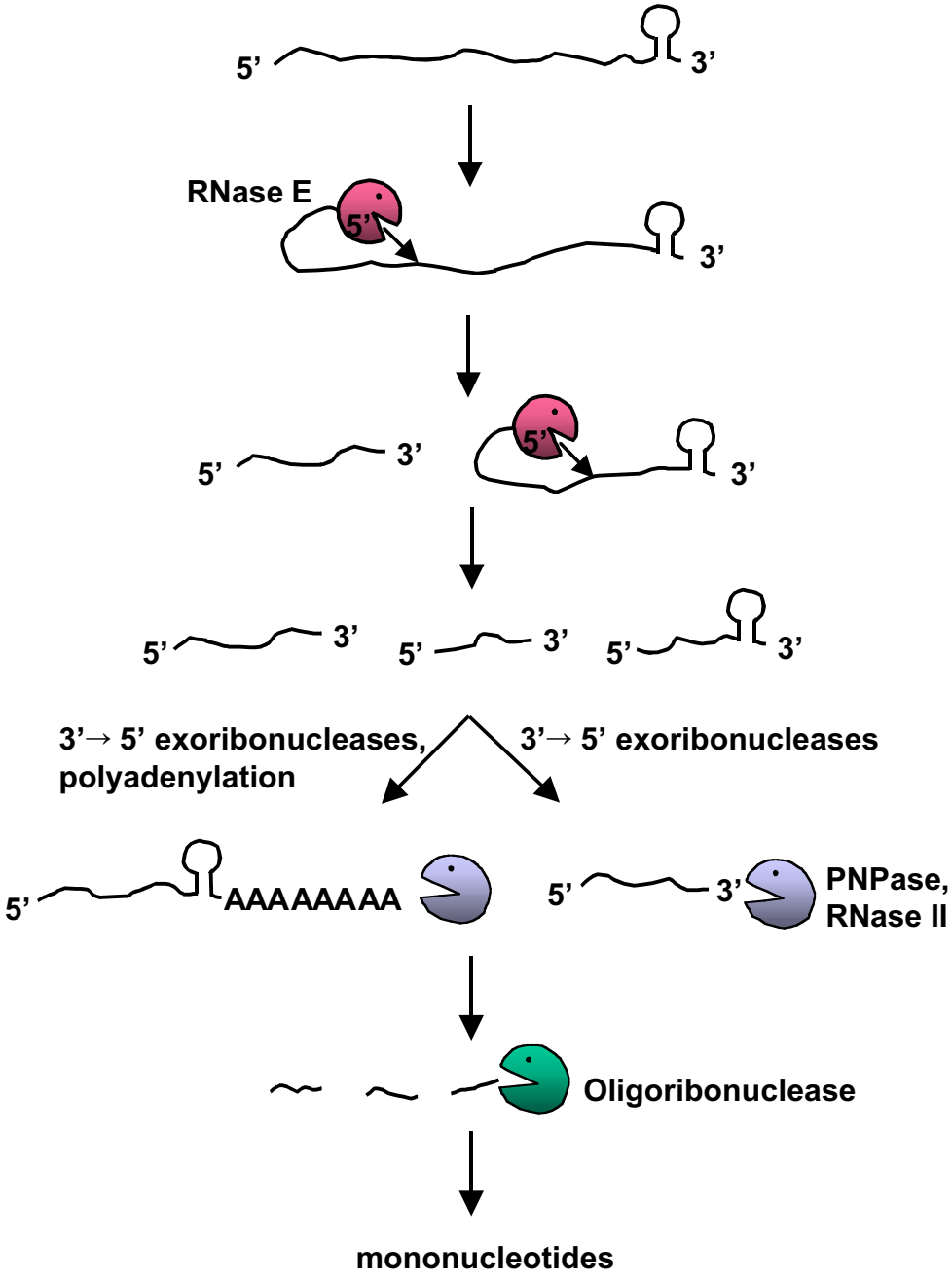

RhlB
enolase
PNPase
S1 domain
RNase E
N
C
1
35
125
498
597
684
734
738
739
844
845
1045
1061
Catalytic Region
ARRBS
Degradosome
scaffolding region

Figure 1.3. A general scheme for the degradation of an mRNA. RNase E makes the initial cleavage on the mRNA by first binding to the triphosphorylated 5' end followed by cleavage at a proximal site. Because RNase E prefers an RNA with a 5' monophosphorylated end over one with a 5' triphosphorylated terminus, multiple rounds of endoribonucleolytic cleavage following the initial cleavage occur more rapidly than the initial cleavage step, generating additional decay intermediates. The net effect of these successive RNase E cleavages is the 5' to 3' direction of decay frequently seen for mRNAs. These intermediates are subsequently directly degraded by 3' to 5' exoribonucleases such as PNPase or RNase II. Alternatively, the intermediates can undergo multiple rounds of polyadenylation and 3' to 5' exoribonucleolytic degradation to remove secondary structures at the 3' terminus. Because neither PNPase nor RNase II can degrade very short oligoribonucleotides, oligoribonuclease is responsible for their final breakdown into mononucleotides.


5’
3’
RNase E
5’
3’
5’
3’
5’
3’
5’
3’
5’
3’
5’
3’
3’→ 5’ exoribonucleases,
polyadenylation
3’→ 5’ exoribonucleases
5’
AAAAAAAA
5’
3’
PNPase,
RNase II
Oligoribonuclease
mononucleotides

CHAPTER 2

ANALYSIS OF mRNA DECAY AND rRNA PROCESSING IN *ESCHERICHIA COLI* IN THE ABSENCE OF RNASE E-BASED DEGRADOSOME ASSEMBLY[1]

---

[1]Ow, M. C., Q. Liu, and S. R. Kushner. 2000. Molecular Microbiology 38: 854-866. Reprinted here with permission of publisher.

ABSTRACT

We demonstrate here that the assembly of the RNase E-based degradosome of *Escherichia coli* is not required for normal mRNA decay *in vivo*. In contrast, deletion of the arginine-rich RNA binding site (ARRBS) from the RNase E protein slightly impairs mRNA decay. When both the degradosome scaffold region and the ARRBS are missing, mRNA decay is dramatically slowed but 9S rRNA processing is almost normal. An extensive RNase E truncation mutation (*rneΔ610*) had a more pronounced mRNA decay defect at 37°C than the temperature-sensitive *rne-1* allele at 44°C. Taken together, these data suggest that the inviability associated with inactivation of RNase E is not related to defects in either mRNA decay or rRNA processing.

INTRODUCTION

Mutations in *rne*, the structural gene for RNase E in *Escherichia coli*, lead to alterations in the rate of decay of both pulse-labeled RNA (Ono and Kuwano, 1979; Arraiano *et al*., 1988) and specific transcripts (Arraiano *et al*., 1988; Mudd *et al*., 1990; Mackie, 1991; Yajnik and Godson, 1993). Extensive biochemical and immunological experiments have demonstrated that the RNase E protein is part of a multiprotein complex, called the degradosome, that also contains the 3' to 5' exoribonuclease polynucleotide phosphorylase (PNPase), the DEAD-box RhlB RNA helicase and enolase (Carpousis *et al*., 1994; Py *et al*., 1994; 1996; Miczak *et al*., 1996). Analysis of various truncated RNase E proteins has established that the carboxy-terminus (amino acids 734-1045) serves as the scaffold for the association of PNPase, RhlB, and enolase (Kido *et al*., 1996; Vanzo *et al*., 1998). Recent work has shown that, *in vitro*, a purified degradosome preparation is sufficient to degrade mRNA (Coburn and Mackie, 1998; Coburn *et al*., 1999).

Although these results have provided support for the hypothesis that the RNase E-containing degradosome is a major player in mRNA decay (Barlow *et al*., 1998; Rauhut and Klug, 1999), no detailed analysis has yet been carried out to determine if the formation of this multiprotein complex is necessary for normal mRNA decay *in vivo*. The fact that

RNase E is a very large protein (1061 amino acids) (Casarégola *et al*., 1992; 1994), composed of three distinct domains (catalytic, arginine-rich RNA binding site [ARRBS] and scaffolding region) (McDowall and Cohen, 1996), makes such an analysis even more important. For example, it has been shown that the *mukB* suppressor mutations first identified by Kido *et al*. (1996) are actually *rne* alleles encoding RNase E proteins lacking either 469 or 477 amino acids from the carboxy-terminal region. Although recent experiments have suggested that such alleles lead to the stabilization of two specific transcripts (López *et al*., 1999), these mutations result in the removal of both the degradosome scaffolding region and the ARRBS from the RNase E protein. Furthermore, the fact that only the N-terminal region of RNase E seems to be conserved in a variety of bacterial species (Kaberdin *et al*., 1998), suggests that degradosome assembly may not be essential for mRNA decay.

For example, it has been known for some time that a shortened form of RNase E (*rneΔ225*), missing 217 amino acids at the carboxy terminus, complements both the *rne-1* and *rne-3071* temperature-sensitive mutants in a *recA* genetic background (Claverie-Martin *et al*., 1989). Since RNase E may function *in vivo* as a dimer (Mackie *et al*., 1997; Coburn and Mackie, 1999), the interpretation of these complementation results is more complicated because both the *rne-1* and *rne-3071* alleles lead to the synthesis of full-length RNase E proteins that retain partial activity. Thus, even though they are complemented by the truncated *rneΔ225* gene (Claverie-Martin *et al*., 1989), it is not possible to exclude the possibility that complementation arises from the formation of a mixed dimer containing one truncated subunit (*rneΔ225*) and one mutant subunit (*rne-1* or *rne-3071*) to which PNPase, RhlB and enolase could associate. In addition, studies of truncated RNase E proteins on plasmids are complicated by the high level of homologous recombination that occurs between the plasmid and chromosomal *rne* genes (Claverie-Martin *et al*., 1989).

In order to circumvent these problems, we report here the construction of an extensive deletion of the chromosomal gene (*rneΔ1018::bla*) in which the ribosome binding site and the first 1018 amino acids of the RNase E coding sequence were replaced with an

ampicillin resistance (Ap^r) cassette. We subsequently complemented this deletion mutation with a series of low-copy-number plasmids carrying a variety of truncated *rne* genes that contained either deletions of 217 to approximately 1034 amino acids from the carboxy-terminus or 91 amino acids within the ARRBS. By determining the half-lives of a number of transcripts, we show that prevention of degradosome assembly has no significant effect on mRNA decay. In contrast, deletion of the ARRBS leads to a small defect in mRNA decay. However, if both regions were deleted, mRNA decay was more severely inhibited than observed in a *rne-1* mutant at either 37°C or 44°C. One truncated RNase E protein (RneΔ610) missing both the degradosome scaffolding region and the ARRBS led to significantly impaired mRNA decay but processed 9S rRNA almost as effectively as the wild-type protein both *in vivo* and *in vitro*.

RESULTS

Construction of a chromosomal *rne* deletion

To generate a chromosomal *rne* deletion, we used a modification of the gene replacement method described by Hamilton *et al*. (1989). In this case a *Xmn*I-*Xmn*I DNA fragment that removed 212 nt of the 5' UTR, the ribosome binding site and the first 1018 amino acids of the RNase E coding sequence was replaced by a DNA fragment carrying the Ap^r gene *bla* to generate the *rneΔ1018::bla* allele (Fig. 2.1, see Experimental Procedures). Subsequently, cointegrants of pQLK61 (containing *rneΔ1018::bla* in pMAK705) in MC1061 were resolved in the presence of a low-copy-number plasmid (pQLK26, rne^+ Km^r carrying a wild-type copy of the *rne* gene. Several resolved cointegrants were obtained that contained the *rne* deletion (*rneΔ1018::bla*) inserted in the chromosome as judged by PCR analysis. The *rneΔ1018::bla* allele was then transduced into an MG1693 (*thyA715*) derivative carrying pQLK26 (rne^+ Km^r) by cotransduction with *pyrC*. Following this step, a *recA56* allele was introduced by co-transduction to prevent homologous recombination between the residual *rne* sequences in the chromosome and those carried on any resident plasmid. Both PCR and Southern blot analysis (data not shown) were employed to confirm

the absence of the chromosomal *rne* gene in SK9705 (*rneΔ1018::bla recA56*/ pQLK26 [*rne*$^+$ Km^r]).

Displacement of the wild-type *rne* gene with the various truncations

Previous work has shown that the RNase E protein consists of three distinct domains: the catalytic region (amino acids 1-500), the ARRBS (amino acids 597-684); and the degradosome scaffolding region (amino acids 734-1045) (Taraseviciene *et al*., 1995; McDowall and Cohen, 1996; Vanzo *et al*., 1998) (Fig. 2.2). In order to determine the importance of these regions for normal mRNA decay, we constructed a series of *rne* deletion mutations (Fig. 2.2), in which either progressive amounts of the carboxy-terminus and/or the ARRBS region were deleted. The *rneΔ91, rneΔ374* and *rneΔ610* alleles (Fig. 2.2) retained the normal translation stop codon while the other constructs contained *rne* sequences fused to vector DNA (Fig. 2.2).

Subsequently, plasmid incompatibility was used to displace the wild-type *rne* gene, carried on either pQLK26 (Km^r or pSBK1 (Cm^r), with the truncated *rne* constructions. Accordingly, either SK9705 (*rneΔ1018::bla*/ pQLK26 [*rne*$^+$ Km^r]) or SK9714 (*rneΔ1018:bla*/ pSBK1 [*rne*$^+$ Cm^r]) was transformed with plasmids carrying the various deletion mutations. In the case of pSYK3 (*rneΔ831* Km^r) and pSYK4 (*rneΔ1034* Km^r), extended selection for Km^r did not lead to the loss of the resident plasmid (pSBK1 Cm^r) in SK9714. In contrast, pMOK17 (*rneΔ91*), pFMK33 (*rneΔ225*), pMOK16 (*rneΔ374*), pSYK1(*rneΔ508*), and pMOK15 (*rneΔ610*) all successfully displaced either pQLK26 or pSBK1.

Growth properties of strains carrying *rne* truncations

All of the deletion plasmids that successfully displaced the wild-type *rne* gene in the *rneΔ1018::bla* strain supported cell viability at both 30^{o}C and 37^{o}C (Table 2.1). The *rneΔ91* strain had a generation time that was almost identical to the wild-type control while the *rneΔ225* and *rneΔ374* mutants had doubling times that were slightly reduced (Table

2.1). In the case of *rneΔ508* and *rneΔ610* alleles, the growth rates were noticeably slower than the wild-type control and the *rneΔ225, rneΔ91*, and *rneΔ374* mutants but faster than the *rne-1* strain (Table 2.1). At 44ºC, neither the *rneΔ508* nor *rneΔ610* alleles could support growth on Luria agar plates (Fig. 2.3). In L broth liquid cultures, growth ceased more rapidly in a *rne-1* strain compared to the *rneΔ610* mutant (Table 2.1). The loss of viable cells at 44ºC in liquid cultures was comparable in the *rne-1* and *rneΔ610* strains (data not shown).

Analysis of degradosome formation in strains carrying *rne* truncation mutations

To assess whether the degradosome assembled in strains carrying the various RNase E truncations described in Fig. 2.2, we first carried out an immunoprecipitation experiment using a PNPase antibody to precipitate the multiprotein complexes. This experiment showed that the interaction between RNase E and PNPase was abolished in strains carrying *rneΔ225*, *rneΔ374,* and *rneΔ610* (Fig. 2.4, lanes 8, 16, 20) but not in *rne*$^+$ or *rneΔ91* (Fig. 2.4, lanes 4, 12). Similarly, the association of RhlB helicase with the degradosome was detected in the *rne*$^+$and *rneΔ91* (Fig. 2.4, lanes 4, 12) but was not observed in the *rneΔ225, rneΔ374*, or *rneΔ610* mutants (Fig. 2.4, lanes 8, 16, 20). Comparable results were obtained when the immunoprecipitation step was carried out using an antibody derived against RNase E (data not shown). These data demonstrated that strains carrying the *rneΔ225*, *rneΔ374*, and *rneΔ610* alleles did not support degradosome assembly while strains carrying either *rne*$^+$ or *rneΔ91* did. These results were in agreement with the observations of Vanzo *et al*. (1998).

mRNA decay in the absence of degradosome formation

To establish whether mRNA decay rates were affected in strains in which the degradosome was not assembled, we first determined the half-lives of a variety of specific transcripts (*trxA, lpp, rpsT, rplY, ompA, pnp*, and *rpsO*) in the *rneΔ225* mutant where PNPase cannot bind to RNase E. With the exception of *lpp*, at 37ºC the half-lives of the

other six transcripts were identical to the wild-type control (Table 2.2). The *lpp* transcript actually had a shorter half-life when PNPase was not associated with the degradosome (Table 2.2). When we repeated the half-life experiments in the *rneΔ374* mutant, a strain in which no components of the degradosome associate with RNase E (Fig. 2.2; Vanzo *et al*., 1998), we again obtained numbers that were comparable to the wild-type control (Table 2.2). Furthermore, the decay profiles in the *rneΔ225* and *rneΔ374* alleles were identical to those observed in the wild-type control (data not shown).

mRNA decay is slightly impaired in the absence of the arginine-rich RNA binding site (ARRBS)

To determine if the arginine-rich RNA binding site (ARRBS) was important for mRNA decay, we constructed an in-frame deletion mutation (*rneΔ91*) which still permitted the normal assembly of the degradosome (Fig. 2.4, lane 12). With this mutation, there were small but reproducible increases in the half-lives of the *trxA, rpsT,* and *rpsO* transcripts that were slightly greater than those observed in mutants (*rneΔ225* and *rneΔ374*) in which degradosome assembly was prevented (Table 2.2).

mRNA decay is seriously impaired in the absence of both the ARRBS and the degradosome scaffolding region

Since the deletion of either the degradosome scaffolding region or the ARRBS did not have a major impact on mRNA decay at 37ºC, we examined mRNA decay in strains carrying more extensive deletions (*rneΔ508* and *rneΔ610*) that removed both portions of the RNase E protein. In the case of *rneΔ508,* the C-terminus of the RNase E protein was fused to approximately 60 amino acids of the C-terminus of the tetracycline resistance protein, whereas in *rneΔ610*, the first 427 amino acids of the protein were fused in-frame to the last 25 amino acids of the carboxy terminus (Fig. 2.2). In both of these strains the half-lives of the *trxA* (Table 2.2) and *rpsT* (Table 2.2, Fig. 2.5) transcripts increased by almost two-fold

compared to the wild-type control. Furthermore, the half-lives were significantly longer than those observed in a *rne-1* control at 37ºC (Table 2.2, Fig. 2.5).

We also compared the half-lives of the *lpp*, *pnp*, and *rpsO* transcripts in *rneΔ610*, *rne-1* and wild-type strains. While the half-life of the *lpp* transcript did not differ significantly among the three strains, both the *pnp*, and *rpsO* transcripts were dramatically stabilized in the *rneΔ610* strain (Table 2.2). In the case of *pnp,* there was a 5-fold increase in the half-life in the *rneΔ610* mutant. For the *rpsO* gene, two transcripts were detected on 6% polyacrylamide gels (Fig. 2.5). *rpsO1* represents an mRNA that terminates at the downstream RNase III cleavage site while *rpsO2* arises from a transcript that either ends at the normal Rho-independent transcription terminator or by processing of *rpsO1* either by RNase E at the M site or exonucleolytic decay (Hajnsdorf *et al*., 1996). In wild-type *E. coli* these two species decayed at slightly different rates (Table 2.2, Fig. 2.5). In both the *rne-1* and *rneΔ610* mutants the half-life of the *rpsO1* transcript increased approximately 2.5-fold (Table 2.2, Fig. 2.5). In striking contrast, the half-life of *rpsO2* increased over 11-fold in the *rneΔ610* strain but less than two-fold in the *rne-1* mutant (Table 2.2, Fig. 2.5).

Decay of some mRNAS at 44ºC is more defective in an *rneΔ610* strain than in an *rne-1* mutant

Although mRNA decay in *rneΔ610* at 37ºC was more defective than in *rne-1*, the fact that the *rne-1* allele encodes a temperature-sensitive enzyme (Mudd *et al*., 1990; Babitzke and Kushner, 1991) led us to compare the half-lives of a number of transcripts at 44ºC. As expected, the half-lives of most of the transcripts tested (*trxA, lpp, rpsT,* and *pnp*) decreased in the wild-type strain at 44ºC compared to those observed at 37ºC (Table 2.3). All transcripts except *lpp* showed an increased half-life at 44ºC in the *rneΔ610* strain compared to the *rne-1* mutant (Table 2.3). With the *rpsT*, *pnp*, and *rpsO* transcripts, the half-lives increased between 9-12 fold in the *rneΔ610* mutant compared to 1.3-3.5 fold in the *rne-1* strain (Table 2.3). The behavior of the *lpp* transcript was somewhat surprising because while it decayed more slowly in the *rne-1* strain (8.0 min, wild-type; 13 min, *rne-1*),

only a marginal increase was observed with the *rneΔ610* allele (Table 2.3). Also of interest was the apparent differential effect that the temperature shift had on particular transcripts in both the *rne-1* and *rneΔ610* strains. For example, the half-life of the *trxA* mRNA decreased in both mutants at 44ºC compared to 37ºC (Tables 2.2, 2.3), while the *rpsT* half-life increased almost 2-fold under the same conditions. In the case of *pnp* the half-life decreased by 2-fold in the *rne-1* strain but increased in the *rneΔ610* mutant (Tables 2.2, 2.3).

Truncation of the RNase E protein interferes with autoregulation

Expression of the RNase E protein is autoregulated in part through the interactions of the protein with the 5' UTR of its transcript (Jain and Belasco, 1995; Diwa *et al*., 2000). Thus, in our experiments RNase E autoregulation was observed when comparing the level of the RNase E protein in MG1693 versus SK9714, a situation in which there were 6-8 copies of the rne^+ gene but only a 2-fold increase in the amount of protein (Fig. 2.6, lanes 1, 2). More RNase E protein (Fig. 2.6, lane 3), as well as transcript (data not shown), was observed in the *rne-1* strain at 37ºC presumably because the partially impaired Rne-1 protein cannot effectively degrade its own transcript. Similar results with the *rne-1* allele were observed by Mudd and Higgins (1993). Surprisingly, even though the *rneΔ610* construct contained an intact 5' UTR, we observed at least a 20-fold increase in the level of the truncated protein compared to the SK9714 wild-type control (Fig. 2.6, lanes 2, 8). This phenomenon was also observed at the transcript level where there was an almost 6-fold increase in the steady-state of the *rneΔ610* message (data not shown). There were also slightly increased levels of RNase E protein in both the *rneΔ91* and *rneΔ374* strains (Fig. 2.6, lanes 4, 6). In contrast, Jiang *et al*. (2000) only observed an 8-fold increase in protein level with an RNase truncation that contained the first 498 amino acids of the protein.

Truncated RNase E proteins have altered cleavage efficiency on the *rne* transcript

The data presented in Tables 2.2 and 2.3 as well as Figs. 2.5 and 2.6 indicated that the RNase E protein encoded by the *rneΔ610* mutation might have modified cleavage efficiency on mRNA transcripts compared to the Rne-1 enzyme. For example, since the 5' UTR of the *rne* transcript contains at least one RNase E cleavage site (Jain and Belasco, 1995), the loss of autoregulation seen in the *rneΔ610* strain (Fig. 2.6, lane 8) suggested that the RneΔ610 protein might not effectively cleave this site. However, in all the strains tested, the 5' UTR was cleaved 49-50 nucleotides downstream (big arrow) of the transcription start site (small arrow) (Fig. 2.7, lanes 1-7). With the wild-type protein the efficiency of cleavage was such that the ratio of full-length to cleaved transcript was less than one (0.93, Fig. 2.7, lane 1). Similar values were obtained with the RneΔ91 (0.62, Fig. 2.7, lane 3) and RneΔ374 (0.85, Fig. 2.7, lane 5) proteins. In contrast, in the *rneΔ508* (Fig. 2.7, lane 6) and *rneΔ610* (Fig. 2.7, lane 7) strains the ratios were 2.67 and 7.39, respectively, demonstrating a significant loss in the ability to cleave the 5' UTR *in vivo*. At 37ºC, the *rne-1* strain had a ratio of 1.58 (Fig. 2.7, lane 2) which increased to 8.23 at 44ºC (data not shown).

In vivo 9S rRNA processing is not significantly altered in strains carrying truncated RNase E proteins

Since the *rneΔ610* mutant was viable but very defective in mRNA decay at 37ºC (Table 2.2, Fig. 2.5) and inviable at 44ºC (Table 2.1), we sought to determine why it could not survive at the elevated temperature. One possibility was that the RneΔ610 protein failed to function properly in its well-established role of processing the 9S rRNA precursor into its p5S form (Ghora and Apirion, 1978). Accordingly, we used Northern blots to examine steady-state RNA isolated from strains carrying truncated RNase E proteins along with wild-type and *rne-1* controls at 37ºC. Interestingly, 9S rRNA processing was relatively normal in all of the strains (Fig. 2.8, lanes 1-6), although a small amount of larger intermediates in the *rneΔ508* and *rneΔ610* strains was detected upon longer exposure of the autoradiogram (Fig. 2.8, lanes 5, 6). While under the experimental conditions used here the

rne-1 strain processed 9S rRNA normally at 37ºC (Fig. 2.8, lane 2), at 44ºC a significant number of larger processing intermediates were observed (Fig. 2.8, lane 8). In contrast, the processing defect seen with the *rneΔ508* and *rneΔ610* mutations at 44ºC (Fig. 2.8, lanes 11,12) was identical to that observed at 37ºC (Fig. 2.8, lanes 5, 6) and far less than that observed with the *rne-1* strain (Fig. 2.8, lane 8).

We also performed *in vitro* RNase E assays on a 9S rRNA substrate using total protein extracts obtained from the mutant strains. The Rne-1, RneΔ225, and RneΔ374 proteins generated the 5S rRNA from the 9S rRNA precursor as efficiently as the wild-type enzyme at 37ºC (data not shown). The protein extract obtained from the *rneΔ610* strain was approximately six times more effective in converting the 9S rRNA into p5S rRNA as compared to the extract from the wild-type control (data not shown). However, it is important to note that while processing of the 9S rRNA into 5S rRNA seemed more efficient in *rneΔ610*, this strain contains an over 20-fold increase of the mutant RneΔ610 protein (Fig. 2.6). When extracts were obtained from cultures grown for 20 min at 44ºC, the Rne-1 protein had reduced activity on the 9S rRNA substrate, while the activity of the RneΔ610 protein was comparable to what was observed at 37ºC (data not shown).

DISCUSSION

Since the discovery of the interaction of RNase E and polynucleotide phosphorylase (Carpousis *et al*., 1994), it has been generally assumed that the RNase E-based degradosome is probably the primary mechanism by which mRNAs are degraded (Barlow *et al*., 1998; Rauhut and Klug, 1999). In fact, López *et al*. (1999) have suggested that degradosome assembly is necessary for mRNA degradation. However, the results presented above demonstrate that the decay of at least seven transcripts is unaffected in the absence degradosome assembly. Two different RNase E truncations that prevented either the association of PNPase (*rneΔ225)* or PNPase, the RhlB RNA helicase, and enolase (*rneΔ374*) led to mRNA half-lives that were almost identical to the wild-type control (Table 2.2). Since these experiments were carried out in a host strain that was deleted for the

chromosomal copy of the *rne* gene (*rneΔ1018::bla*) and was recombination deficient (*recA56*), the results obtained could not have been affected by some type of interaction between the plasmid encoded *rne* genes and an inactivated chromosomal *rne* locus.

The stabilization of both bulk mRNA and some chimeric transcripts that were seen by López *et al*. (1999) occurred in strains carrying truncated RNase E proteins that were missing both the degradosome scaffold region and the arginine-rich RNA binding site (ARRBS) (Taraseviciene *et al*., 1995; McDowall and Cohen, 1996) through the deletion of either 469 or 477 amino acids from the carboxy-terminus. It thus was of interest that when we constructed an *rne* allele that was only missing the ARRBS but retained the degradosome scaffold region (*rneΔ91*), mRNA decay was slightly impaired compared to both the wild-type control and the *rne* alleles that prevented degradosome assembly (*rneΔ225, rneΔ374*, Table 2.2). Since the N-terminal catalytic domain (amino acids 1-500) must also contain an RNA binding site in order to carry out phosphodiester bond cleavage, what is the function of the ARRBS? One possibility is that it increases the processivity of the enzyme, an idea first suggested by McDowall and Cohen (1996). Coburn and Mackie (1999) have proposed that once RNase E binds to the 5' terminus of a mRNA, it moves processively towards the 3' end. It is this processivity that could account for the very rapid disappearance of a full-length transcript once decay is initiated. If RNase E acted distributively in the absence of the ARRBS, one would predict some slowing in the decay rates of individual transcripts as was in fact observed. However no new decay intermediates were observed.

In contrast, the RNase E truncation that was lacking both the ARRBS and the degradosome scaffold region (*rneΔ610*) had a dramatic effect on mRNA decay (Tables 2.2, 2.3, Fig. 2.5). Besides demonstrating the impact of the ARRBS and the degradosome scaffold region on mRNA decay, the properties of the *rneΔ610* encoded protein provide interesting new insights into other aspects of RNase E function and regulation. Although only 126 amino acids shorter than the RneΔ508 protein, RneΔ610 is present in much high levels *in vivo* (Fig. 2.6, lanes 7, 8) and has altered cleavage efficiency on transcripts such as

rpsO (Fig. 2.5) and *rne* (Fig. 2.7) compared to the temperature-sensitive *rne-1* allele. With regard to its action on the *rpsO* transcripts, it is not clear at this time what accounts for the dramatic stabilization of the *rpsO2* transcript. However, it should be pointed out that no new decay intermediates were observed with any of the *rne* deletion alleles described here.

The data described above with the *rneΔ610* and *rneΔ508* alleles along with the results of López *et al*. (1999) demonstrate clearly that RNase E functions differently in mRNA decay than in the processing of 9S rRNA. Even with the shortest RNase E protein (RneΔ610), processing of 9S rRNA was hardly perturbed at either 37ºC where the cell was viable or at 44ºC where the cell was inviable. In contrast, in the *rne-1* strain, 9S rRNA processing was significantly impaired at 44ºC (Fig. 2.8, lane 8). These results indicate that neither degradosome assembly nor the presence of the ARRBS are necessary for the cleavage of the two RNase E sites within the 9S rRNA precursor. This observation provides further support for the idea that RNase E cleavage sites within the 9S rRNA transcript are different compared to the sites found in the *rne* and *rpsO* transcripts.

The analysis of RNase E protein levels in the various truncation mutants (Fig. 2.6) raises some interesting questions regarding the autoregulation of its synthesis. Jain and Belasco (1995) and Diwa *et al*. (2000) have demonstrated using *rne::lacZ* fusion constructs that the 5' UTR is required for autoregulation to occur. These requirements make sense since the 5' UTR contains RNase E cleavage sites. It was thus interesting that both the *rneΔ91* and *rneΔ374* alleles led to moderate increases in RNase E synthesis compared to the wild-type control (Fig. 2.6, lanes 4, 6). Surprisingly, the *rneΔ610* allele led to a greater than 20-fold increase in protein, even though the 5' UTR was intact. Although there was a 6-fold increase in the steady-state of the *rneΔ610* transcript (data not shown), this change does not seem to be sufficient to explain the very large accumulation of RneΔ610 protein. In addition, the increase in RneΔ610 protein was much larger than the 8-fold increase seen with a 498 amino acid N-terminal fragment (Jiang *et al*., 2000). The lower levels of protein seen with the *rneΔ225* and *rneΔ508* alleles probably arises from the fact that in these constructions the *rne* coding sequence is fused to a portion of the tet^r gene.

Another interesting question regards why RNase E is an essential protein in *E. coli*? While it has been thought that its central role in mRNA decay is the reason that the protein is required for cell viability, our data suggest that this may not be the case. In the first place, the *rneΔ610* allele was able to support cell viability at 30ºC and 37ºC but not at 44ºC. However, at 37ºC the defects in mRNA decay associated with the *rneΔ610* allele (Table 2.2) were greater than those measured in the *rne-1* strain at its nonpermissive temperature (Table 2.3). In addition, at 44ºC the defect in mRNA decay associated with the *rneΔ610* allele was not appreciably more than that observed at 37ºC (Tables 2.2, 2.3), even though the strain was now inviable. While it is possible that we have not chosen the appropriate transcripts to analyze, it is more likely that the inactivation of mRNA decay is not the primary reason why RNase E is essential for cell viability. When coupled with the almost normal processing of 9S rRNA by the *rneΔ610* mutant, it seems possible that RNase E has a function, yet to be identified, that is essential for cell viability.

Finally, it is interesting to speculate on why the degradosome exists in *E. coli*, if it is not required for normal mRNA decay. Clearly the fact that some bacteria contain homologues of the N-terminal region (catalytic) of RNase E but not the degradosome scaffolding region (Kaberdin *et al*., 1998) suggests that endonucleolytic cleavage of mRNAs is a common degradation pathway in bacteria even if degradosome formation is not. While it cannot be ruled out that the normal decay of a few transcripts requires degradosome assembly, a more likely explanation is that the degradosome may have some other function in the cell. In this regard it is worth noting that while the growth properties of the ARRBS deletion (*rneΔ91*) were identical to the wild-type control, the *rneΔ225* and *rneΔ374* mutants grew more slowly at both 37ºC and 44ºC.

EXPERIMENTAL PROCEDURES

Bacterial strains

The bacterial strains used in this work are described in Table 2.4. A chromosomal *rne* deletion was constructed using a modification of the gene replacement method described by Hamilton *et al*. (1989). An *Xmn*I-*Xmn*I fragment, encompassing the ribosome binding site and the first 1018 amino acids of the RNase E coding sequence (Fig. 2.1) was replaced in pQLK16 [contains a 6.2 kb *Pst*I-*Pst*I chromosomal DNA fragment in pWSK29 (Wang and Kushner, 1991)] with a 1.7 kb *Aat*II-*Sac*II fragment isolated from pACYC177 (Chang and Cohen, 1978) carrying the *bla* (Ap^r) gene. This chimeric DNA fragment (*rneΔ1018::bla*) was then transferred into pMAK705 (Hamilton *et al*., 1989), a Cm^r vector carrying a temperature-sensitive origin of replication to form pQLK61. pQLK61 plasmid DNA was transformed into MC1061 at 30ºC to generate SK8416. SK8416 was grown at 30ºC in an overnight standing culture (L broth + 20 μg/ml Cm) and a series of serial dilutions were grown at 44ºC on L agar plates containing 20 μg/ml Cm. One cointegrate that grew at 44ºC (SK8320) was picked for further work. Initial efforts to resolve SK8320 at 30ºC proved unsuccessful. Accordingly, SK8320 was grown and transformed with pQLK26 (*rne*$^+$ Km^r) at 44ºC to yield SK8321. Since both pQLK26 and pQLK61 had the same origin of DNA replication, we hypothesized that when the cointegrate resolved, the pMAK705 derivative (pQLK61) would be lost if we selected for Km^r. Because pQLK26 carried a wild-type copy of the *rne* gene, a resolved cointegrate carrying the *rneΔ1018::bla* allele in the chromosome would still be viable. Accordingly, SK8321 was grown overnight with shaking at 30ºC in L broth containing 50 μg/ml of kanamycin. In the morning the cultures were diluted into fresh L broth and samples were plated on L agar plates + 50 μg/ml Km. This process was repeated every twelve hours. From a culture that had been grown for five days, three Cm^s isolates were obtained from the 200 Km^r colonies that were tested. The presence of the *rneΔ1018*::*bla* allele was confirmed by analyzing chromosomal DNA using PCR. Two of the three isolates contained the *rneΔ1018*::*bla* allele. One

(SK8322) was chosen for further work. The *rneΔ1018*::*bla* allele was subsequently moved into the MG1693 (Arraiano *et al*., 1988) genetic background via P1 transduction by selecting for PyrC$^+$ transductants in a strain (SK9205 *pyrC*::Tn*10*) carrying pQLK26 (*rne*$^+$ Km$^+$) to generate SK9206. The presence of the *rneΔ1018*::*bla* allele in SK9206 was verified by testing for Ap$^+$ and by PCR and Southern blot analysis (data not shown). To prevent homologous recombination between the *rne* gene on the plasmid and small amount of *rne* sequences remaining in the chromosome, SK9206 was subsequently made RecA$^-$ by the introduction of the *recA56* allele from JC10240 (Csonka and Clark, 1980) via P1 transduction to generate SK9705 (*rneΔ1018*::*bla recA56 srl-300*::Tn*10 thyA715* [pQLK26 *rne*$^+$ Km$^+$]).

Plasmids constructions

Plasmid pMOK15 (*rneΔ610*) was constructed by restriction enzyme digestion of the *Pml*I and *Bst*BI sites in the *rne* gene carried on pSBK1 (*rne*$^+$ Cm$^+$). The digested plasmid DNA was gel-purified (Qiagen), treated with mung bean nuclease to remove the 5' overhang resulting from the *Bst*BI cleavage and religated to generate an in-frame deletion. Unexpectedly, the procedure resulted in a plasmid that that been shortened upstream of the *Pml*I site by 173 nucleotides. DNA sequencing confirmed that the larger deletion remained in-frame.

Plasmid pMOK16 (*rneΔ374*) was made by PCR using Platinum *Pfx* DNA polymerase (Life Technologies). A 3.2 kb *rne* fragment up to and including the ARRBS was amplified using primers RNE-1014 (5' CGGTGCTTGAATTCTCTTC-ACATGCGACTG 3') and RNE+2416 (5' CGCCTTCGCTTCTTCTAGAGCCTAACG-TTTATC 3') which contained engineered *Eco*RI and *Xba*I sites as well as a stop codon at the 3' end. This fragment was subsequently cloned into the *Eco*RI and *Xba*I sites in pWSK129 (Wang and Kushner, 1991). Plasmid pMOK17 (*rneΔ91*) was constructed using overlap extension PCR (Ho *et al*., 1989) with primers RNE-1014, RNEARM-B (5' TCGCTTCTTGTTGCGCCTGTTTCGGTGCTGGTTGCTCGGT 3'), RNEARM-C (5'

TCGCTTCTTGTTGCGCCTGTTTCGGTGCTGGTTGCTCGGT 3', and RNE+3628 (5' CATTCTAGATTAGCAAGGATGCCATTC 3') to introduce an in-frame deletion of the ARRBS. The PCR fragment was digested with *Eco*RI and *Xba*I and cloned into the *Eco*RI and *Xba*I sites of pWSK129. The in-frame deletion of the ARRBS was confirmed by DNA sequencing. To make plasmid pMOK13 (*rne-1*) an *Nsi*I/*Mlu*I 3.2 kb *rne* fragment of pSBK1 was replaced with the *Nsi*I/*Mlu*I *rne-1* fragment of pSYK106 which carried the temperature-sensitive allele (S. D. Yancey and S. R. Kushner, unpublished results).

Plasmid displacement

Recipient strains were transformed with an appropriate plasmid and transformants were selected at 37ºC based on the drug marker carried on the incoming plasmid. Individual transformants were then grown for 1-5 days in the presence of the drug whose resistance was encoded by the incoming plasmid. Subsequently, the loss of the resident plasmid was determined by replica plating of 100-500 individual colonies. For some of the experiments described above pQLK26 (*rne*$^+$ Km^r) in SK9705 was displaced with pSBK1 (*rne*$^+$ Cm^r) to generate SK9714. The *rneΔ225, rneΔ508, rneΔ91,* and *rneΔ374* alleles were introduced into the SK9714 host by displacing pSBK1 with either pFMK33, pSYK1 (Claverie-Martin *et al*., 1989), pMOK17, or pMOK16 to generate SK9950 (*rneΔ225*), SK9952 (*rneΔ508*), SK9976 (*rneΔ91*), and SK9971 (*rneΔ374*), respectively. The *rne-1* and *rneΔ610* alleles were introduced into SK9705 by displacement of pQLK26 with either pMOK13 or pMOK15 to create SK9937 (*rne-1*) and SK9957 (*rneΔ610*), respectively. Plasmids pQLK26, pSBK1, pFMK33, pSYK1, pSYK3, pSYK4, pMOK13, pMOK15, pMOK16, and pMOK17 all carried the pSC101 origin of DNA replication.

Growth curves

Cultures were grown at 37ºC with shaking in L broth (supplemented with 50 μg/ml thymine and either 20 μg/ml of chloramphenicol or 50 μg/ml of kanamycin) until they reached a density of 5 x 10^7 cells/ml at which point they were either shifted to 44ºC or kept at 37ºC.

Immunoprecipitations

Cells were grown (100 ml cultures) at 37ºC in L broth (supplemented with 50 μg/ml of thymine and 20 μg/ml of chloramphenicol or 50 μg/ml of kanamycin) with shaking and collected at a cell density of approximately 2 x 10^8 cells/ml. The cell pellets were washed with 50 ml of cold 50 mM Tris, pH 7.5, 100 mM NaCl and resedimented. The resulting pellets were then resuspended in IP buffer (100 mM NaCl, 1% nonidet-40, 0.1% sodium dodecyl sulfate, 0.5% sodium desoxycholate, 50 mM Tris pH 7.5, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride [Sigma], 2 μg/ml aprotinin [Sigma], 0.8 μg/ml leupeptin [Boehringer Mannheim], 0.8 μg/ml pepstatin A [Boehringer Mannheim] and 1 mM EDTA), lysed by sonication with three 30 seconds bursts in an ice bath and centrifuged at 13,000 rpm for 10 min. Protein concentrations were determined by the method of Bradford (Bradford, 1976) with bovine serum albumin as the standard. All immunoprecipitations were done at 4ºC. One and a half mg of each cleared supernatant fraction was added to PNPase antiserum and shaken gently for one hour. Protein complexes were precipitated by the addition of 10 μl of Protein G Plus/Protein A-agarose beads (Oncogene Research Products), followed by gentle agitation for an additional 30 min. The protein/bead pellets were collected and washed four times with IP buffer. After the last wash, the immune complexes were solubilized at 100ºC for 5 min in 30 μl of SDS/polyacrylamide gel loading buffer (60 mM Tris pH 6.8, 2% ß-mercaptoethanol, 2% sodium dodecyl sulfate, 10% glycerol, 0.01% bromophenol blue) and loaded into an 8% SDS/PAGE gel.

Because the RneΔ508, RneΔ610, and the RhlB helicase proteins migrated along with the antibodies in the protein gels, we also used the immunoprecipation procedure described

by Vanzo *et al*. (1998) so that the antibodies would not interfere with the detection of the proteins on a Western blot. Briefly, either PNPase or RNase E antibodies were coupled to Protein A-Sepharose beads (Pharmacia) in CLB buffer (20 mM Na_2HPO_4, 5 mM NaH_2HPO_4, 0.2 mM NaCl, 0.5 mM EDTA), washed in CLB buffer and followed by crosslinking with 1% glutaraldehyde (Sigma) for 1 hr at 4ºC. The beads with the antibodies were added to 0.4-2 mg of a cleared total protein lysate and agitated gently for 1 hr at 4ºC. The samples were washed five times with IP buffer and the beads resuspended in 100 μl of nondenaturing SDS/PAGE loading buffer. The immunocomplexes were solubilized at 55ºC for 15 min. The supernatants were retained and boiled in 2% ß-mercaptoethanol for 5 min and analyzed in an 8% SDS/PAGE gel.

The protein gels were transferred to Immobilon-P transfer membrane (Millipore) by electroblotting and specific proteins were detected using the ECL Western blotting analysis system (Amersham Life Science) according to the manufacturer's instructions. Antibodies for RNase E, PNPase and RhlB helicase were used in the following dilutions, respectively: 1:2000; 1:10000 and 1:1000.

Northern blot analysis

Total RNA preparation and Northern blotting (*trxA*, *lpp*, *rplY*, *rpsT*, and *rpsO*) were performed as described by O'Hara *et al*. (1995). For agarose Northern blots (*ompA*, *pnp*, and *rpsO*) total RNA was denatured and electrophoresed as indicated by Burnett (1997). Northern blots were quantified using a Molecular Dynamics series 400 PhosphorImager. Messenger RNA half-lives were calculated using linear regression analysis.

Primer extension analysis

The primers used for primer extension analysis of the *rne* 5' UTR were QLrneL2 (5' CCATCTACCGGTAAGGACTGC 3') and RNE+240 (5' CCTCACGGTTATCGTCAGCTC 3'). The primers were 5' end-labeled with T4 polynucleotide kinase (New England Biolabs) and purified using a Centri-Spin 20 column

(Princeton Separations) according to the instructions of the manufacturer. Twenty five to 50 μg of total RNA were dried and resuspended in 10 μl of RNase-free water and 2 μl of annealing buffer (1 M KCl, 100 mM Tris-HCl pH 8.3). Two μl of a 5' end-labeled primer (approximately 1 x 10^5 cpm) were added and the samples were heated to 80°C for 5 min, slowly cooled to 42°C and incubated for 2 hrs. An extension mix (AMV reverse transcriptase buffer [Boehringer Mannheim], 160 μM dNTPs, 20 units of RNasin [Promega], and 12.5 units of AMV reverse transcriptase [Boehringer Mannheim]) were added and the samples were further incubated for 1 hr at 42°C. The extension was stopped with 20 mM EDTA, treated with 0.4 mg/ml of RNase A and phenol: chloroform extracted. The cDNA was ethanol precipitated and analyzed in 6% polyacrylamide/7 M urea gels next to sequencing ladders generated from the same primers that were used for the primer extension.

RNase E assays

In vitro RNase E assays were done as described by Babitzke and Kushner (1991). Assays were conducted at 37°C or 44°C using 0.5 μg of total protein extract. The RNase E proteins in *rne-1, rneΔ508*, and *rneΔ610* were inactivated by shifting 37°C exponentially growing cultures (1 x 10^8 cells/ml) to 44°C for 70 minutes prior to extract isolation. Samples were analyzed in 6% polyacrylamide/7 M urea gels.

ACKNOWLEDGEMENTS

This work was supported in part by NIH grants (GM28760 and GM52210) to S.R.K. We thank Stephanie Bundy for technical assistance, A. J. Carpousis for graciously providing antibodies and B. Mohanty for critical reading of the manuscript.

REFERENCES

Arraiano, C.M., S. D. Yancey, and S. R. Kushner. 1988. Stabilization of discrete mRNA breakdown products in *ams pnp rnb* multiple mutants of *Escherichia coli* K-12. J. Bacteriol. 170: 4625-4633.

Babitzke, P., and S. R. Kushner. 1991. The Ams (altered mRNA stability) protein and ribonuclease E are encoded by the same structural gene of *Escherichia coli*. Proc. Natl. Acad. Sci. USA 88: 1-5.

Barlow, T., M. Berkmen, D. Georgellis, L. Bayr, S. Arvidson, and A. Von Gabain. 1998. RNase E, the major player in mRNA degradation, is down-regulated in *Escherichia coli* during a transient growth retardation (Diauxic lag). Biol. Chem. 379: 33-38.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Burnett, W.V. 1997. Northern blotting of RNA denatured in glyoxal without buffer recirculation. BioTechn. 22: 668-671.

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

Casadaban, M. J., and S. N. Cohen. 1980. Analysis of the gene control signals by DNA fusion and cloning in Escherichia coli. J. Mol. Biol. 138: 179-207.

Casarégola, S., A. Jacq, D. Laoudj, G. Mcgurk, S. Margarson, M. Tempête, V. Norris, and I. B. Holland. 1992. Cloning and analysis of the entire *Escherichia coli ams* gene. *ams* is identical to *hmp1* and encodes a 114 kDa protein that migrates as a 180 kDa protein. J. Mol. Biol. 238: 867.

Casarégola, S., A. Jacq, D. Laoudj, G. Mcgurk, S. Margarson, M. Tempête, V. Norris, and I. B. Holland. 1994. Cloning and analysis of the entire *Escherichia coli ams* gene. *ams* is identical to *hmp-1* and encodes a 114 kDa protein that migrates as a 180 kDa protein. J. Mol. Biol. 228: 30-40.

Chang, A.C.Y., and S. N. Cohen 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic mini-plasmid. J. Bacteriol. 134: 1141-1156.

Claverie-Martin, F., M. R. Diaz-Torres, S. D. Yancey, and S. R. Kushner. 1989. Cloning of the altered mRNA stability (*ams*) gene of *Escherichia coli* K-12. J. Bacteriol. 171: 5479-5486.

Coburn, G.A., and G. A. Mackie. 1998. Reconstitution of the degradation of the mRNA for ribosomal protein S20 with purified enzymes. J. Mol. Biol. 279: 1061-1074.

Coburn, G.A., and G. A. Mackie. 1999. Degradation of mRNA in *Escherichia coli*: An old problem with some new twists. Prog. Nucl. Acid Res. 62: 55-108.

Coburn, G.A., X. Miao, D. J. Briant, and G. A. Mackie. 1999. Reconstitution of a minimal RNA degradosome demonstrates functional coordination between a 3' exonuclease and a DEAD-box RNA helicase. Genes Dev. 13: 2594-2603.

Csonka, L.N., and A. J. Clark. 1980. Construction of an Hfr strain useful for transferring *recA* mutations between *Escherichia coli* strains. J. Bacteriol. 143: 529-530.

Diwa, A., A. L. Bricker, C. Jain, and J. G. Belasco. 2000. An evolutionarily conserved RNA stem loop functions as a sensor that directs feedback regulation of RNase E gene expression. Genes Dev. 14: 1249-1260.

Ghora, B.K., and D. Apirion. 1978. Structural analysis and *in vitro* processing to p5 (5S) rRNA of a 9S RNA molecule isolated from an *rne* mutant of *E. coli*. Cell 15: 1055-1066.

Hajnsdorf, E., F. Braun, J. Haugel-Nielsen, J. Le Derout, and P. Régnier. 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.

Hamilton, C.M., M. Aldea, B. K. Washburn, P. Babitzke, and S. R. Kushner. 1989. A new method for generating deletions and gene replacements in *Escherichia coli*. J. Bacteriol. 171: 4617-4622.

Ho, S.N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77: 51-59.

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.

Jiang, X., A. Diwa, and J. G. Belasco. 2000. Regions of RNase E important for 5'-end-dependent RNA cleavage and autoregulated synthesis. J. Bacteriol. 182: 2468-2475.

Kaberdin, V.R., A. Miczak, J. S. Jakobsen, S. Lin-Chao, K. J. McDowall, and A. von Gabain. 1998. The endoribionucleolytic N-terminal half of *Escherichia coli* RNase E is evolutionarily conserved in *Synechocystis* sp. and other bacteria but not the C-terminal half, which is sufficient for degradosome assembly. Proc. Natl. Acad. Sci. USA 95: 11637-11642.

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

López, P.J., I. Marchand, I, S. A. Joyce, and M. Dreyfus. 1999. The C-terminal half of RNase E, which organizes the *Escherichia coli* degradosome, participates in mRNA degradation but not rRNA processing *in vivo*. Mol. Microbiol. 33: 188-199.

Mackie, G.A. 1991. Specific endonucleolytic cleavage of the mRNA for ribosomal protein S20 of *Escherichia coli* requires the products of the *ams* gene *in vivo* and *in vitro*. J Bacteriol 173: 2488-2497.

Mackie, G.A., J. L. Genereaux, and S. K. Masterman. 1997. Modulation of the activity of RNase E *in vitro* by RNA sequences and secondary structures 5' to cleavage sites. J. Biol. Chem. 272: 609-616.

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 motif. J. Mol. Biol. 255: 349-355.

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.

Mudd, E.A., A. J. Carpousis, and H. M. Krisch. 1990. *Escherichia coli* RNAse E has a role in the decay of bacteriophage T4 mRNA. Genes Dev. 4: 873-881.

Mudd, E.A., and C. F. Higgins. 1993. *Escherichia coli* endoribonuclease RNase E: autoregulation of expression and site-specific cleavage of mRNA. Mol. Microbiol. 3: 557-568.

O'Hara, E.B., J. A. Chekanova, C. A. Ingle, Z. R. Kushner, E. Peters, and S. R. Kushner. 1995. Polyadenylylation helps regulate mRNA decay in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 92: 1807-1811.

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

Py, B., H. Causton, E. A. Mudd, and C. F. Higgins. 1994. A protein complex mediating mRNA degradation in *Escherichia coli*. Mol. Microbiol. 14: 717-729.

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.

Rauhut, R., and G. Klug. 1999. mRNA degradation in bacteria. FEMS Microbiol. Rev. 23: 353-370.

Taraseviciene, L., G. R. Björk, and B. E. Uhlin. 1995. Evidence for a 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, L. C. Raynal, H. M. Krisch, and A. J. Carpousis. 1998. Ribonuclease E organizes the protein interactions in the *Escherichia coli* RNA degradosome. Genes Dev. 12: 2770-2781.

Wang, R.F., and S. R. Kushner. 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and expression in *Escherichia coli*. Gene 100: 195-199.

Yajnik, V., and G. N. Godson. 1993. Selective decay of *Escherichia coli dnaG* messenger RNA is initiated by RNase E. J. Biol. Chem. 268: 13253-13260.

Table 2.1. Generation time of strains carrying various *rne* alleles.

Strain	Genotype[a]	Generation Time (min)	
		37°C	44°C
SK9714	*rneΔ1018* / *rne*$^+$	27	25
SK9937	*rneΔ1018* / *rne-1*	45	TS[b]
SK9976	*rneΔ1018* / *rneΔ91*	30	36
SK9950	*rneΔ1018* / *rneΔ225*	33	38
SK9971	*rneΔ1018* / *rneΔ374*	33	38
SK9952	*rneΔ1018* / *rneΔ508*	39	TS[c]
SK9957	*rneΔ1018* / *rneΔ610*	39	TS[c]

[a]Chromosomal genotype / plasmid genotype.

[b]TS, temperature-sensitive. Growth in Luria broth ceased within 120 minutes after shift to 44°C.

[c]TS, temperature-sensitive. Growth in Luria broth cease within 240 minutes after shift to 44°C.

Table 2.2. mRNA half-lives at 37°C in the presence of various RNase E proteins.

Transcript	Half-life (min)						
	rne^{+}	*rneΔ225*	*rneΔ374*	*rneΔ91*	*rneΔ508*	*rneΔ610*	*rne-1*
trxA	1.9 ± 0.3	1.9 ± 0.2	2.2 ± 0.3	2.4 ± 0.1	3.3 ± 0.1	3.3 ± 0.3	2.6 ± 0.3
lpp	13.3 ± 2.1	9.8 ± 1.9	ND[a]	ND	ND	15.1 ± 1.1	13.7 ± 1.0
rpsT[b]	1.6 ± 0.2	1.5 ± 0.4	1.6 ± 0.2	1.8 ± 0.4	3.7 ± 0.5	4.7 ± 0.7	2.0 ± 0.5
rplY	3.5 ± 0.3	3.7 ± 0.2	ND	ND	ND	ND	ND
ompA	10.6 ± 1.2	10.4 ± 1.2	6.2 ± 0.6	ND	ND	ND	ND
pnp	1.0 ± 0.1	1.2[c]	1.3 ± 0.2	ND	ND	5.4 ± 1.0	1.7 ± 0.1
rpsO1	1.0 ± 0.2	ND	1.5 ± 0.2	1.8 ± 0.4	ND	2.4 ± 0.4	2.7 ± 0.8

rpsO2	1.5 ± 0.1	1.5[c]	1.7 ± 0.2	2.1 ± 0.1	ND	17.3 ± 2.9	2.7 ± 0.8

[a]Not determined.

[b]Represents the average of the two bands marked with arrows.

[c]These half-life determinations were only done once.

Half-lives were determined as described in Experimental Procedures and represent the average of at least 3 independent determinations unless noted otherwise.

Table 2.3. mRNA half-lives at 44°C in the presence of various RNase E proteins.

Transcript	Half-life (min)[a]		
	rne^{+}	*rne-1*	*rne*D*610*
trxA	1.2 ± 0.1(1.9) [b]	1.3 ± 0.2 (2.6) [b]	1.8 ± 0.3 (3.3) [b]
lpp	8.0 ± 1.7 (13.3) [b]	13 ± 2.4 (13.7) [b]	9.2 ± 2.3 (15.1) [b]
rpsT	1.0 ± 0.1 (1.6) [b]	3.3 ± 0.3 (2.0) [b]	9.2 ± 2.3 (4.7) [b]
pnp	0.6 ± 0.1(1.0) [b]	0.8 ± 0.2 (1.7) [b]	7.5 ± 0.9 (5.4) [b]
rpsO[c]	1.6 ± 0.4	5.6 ± 0.5	15.9 ± 1.1

[a] Half-lives were measured as described in the Experimental Procedures and represent the average of at least 3 independent determinations.

[b] Numbers in parenthesis are the comparable half-lives determined at 37°C as shown in Table 2.2. They are included here to make it to compare the half-lives determined at both temperatures.

[c] This experiment was carried out in a 1.25% agarose gel such that the *rpsO1* and *rpsO2* transcripts migrated as a single band. As a result the *rpsO* half-lives here may not be directly comparable to those determined in Table 2.2.

Table 2.4. Bacterial strains used.

Strain	Genotype	Source or reference
JC10240	*recA56 srl-300::*Tn*10* Tc^r	Csonka and Clark, 1980
MC1061[a]	*relA1 spoT1 hsdR2 mcrB9999 rpsL*	Casadaban and Cohen, 1980
MG1693	*thyA715 rph-1*	*E. coli* Genetic Stock Center
SK5664	*thyA715 rph-1 pyrC::*Tn*10* Tc^r	This laboratory
SK8416	MC1061/ pQLK61 [*rneΔ1018::bla* Cm^r]	This study
SK8320	MC1061 with pQLK61 integrated into the chromosome	This study
SK8321	SK8320 carrying pQLK26 [*rne*$^+$ Km^r]	This study
SK8322	MC1061 with *rneΔ1018::bla* in chromosome and pQLK26 [*rne*$^+$ Km^r]	This study
SK9205	*thyA715 rph-1 pyrC::*Tn*10* Tc^r/ pQLK26 [*rne*$^+$ Km^r]	This study
SK9206	*rneΔ1018*::*bla thyA715 rph-1*/ pQLK26 [*rne*$^+$ Km^r]	This study
SK9705	*rneΔ1018*::*bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pQLK26 [*rne*$^+$ Km^r]	This study

SK9714	*rneΔ1018*::*bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pSBK1 [*rne*$^+$ Cm^r]	This study
SK9937	*rneΔ1018*::*bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pMOK13 [*rne-1* Cm^r]	This study
SK9950	*rneΔ1018*::*bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pFMK33 [*rneΔ225* Km^r]	This study
SK9952	*rneΔ1018*::*bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pSYK1 [*rneΔ508* Km^r]	This study
SK9957	*rneΔ1018*::*bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pMOK15 [*rneΔ610* Cm^r]	This study
SK9971	*rneΔ1018*::*bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pMOK16 [*rneΔ374* Km^r]	This study
SK9976	*rneΔ1018*::*bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pMOK17 [*rneΔ91* Km^r]	This study

[a] MC1061 has proven to be an efficient host strain for carrying out gene displacements using the method of Hamilton *et al*. (1989).

Figure 2.1. Description of *rneΔ1018::bla*. A schematic structure of the wild-type *rne* gene is presented including the +1 transcription start-site along with the AUG and UAA translation start and stop codons. The full-length RNase E protein contains 1061 amino acids (Casarégola *et al*., 1992; 1994). The *rneΔ1018::bla* allele retains 149 nt of the 5' UTR and 43 amino acids of coding sequence at the 3' end of the protein. The *Xmn*I-*Xmn*I DNA fragment was replaced by a *bla* (Ap^r) cassette as described in Experimental Procedures.

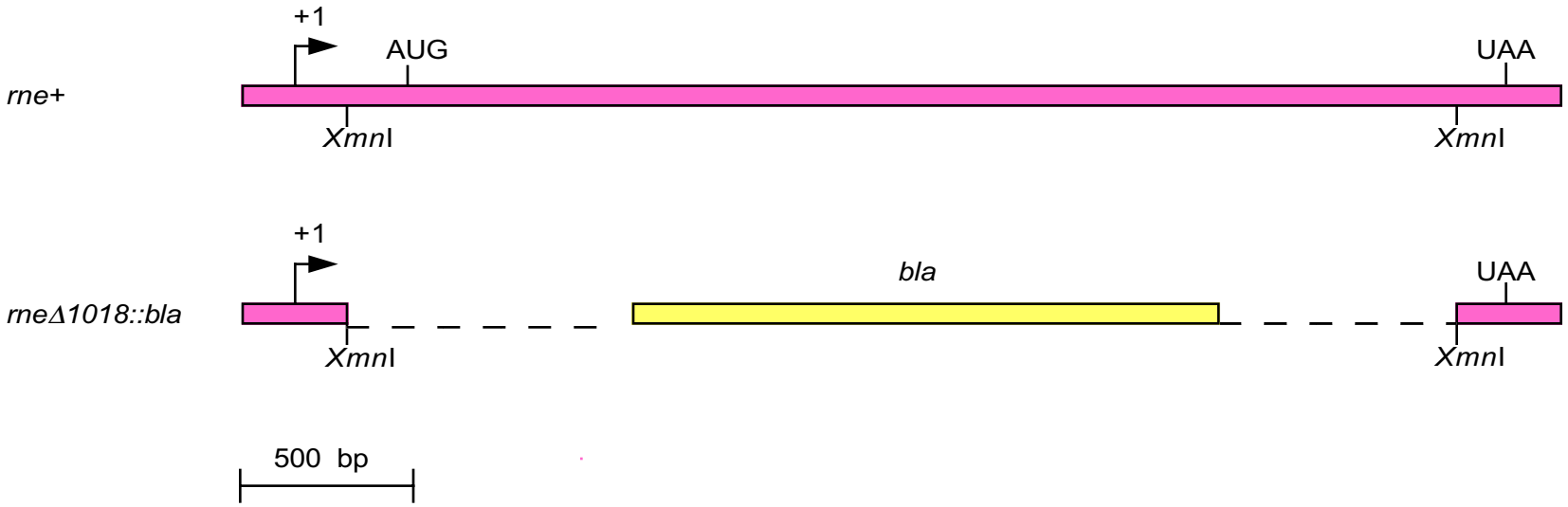

+1
AUG
UAA
rne+
XmnI
XmnI
+1
bla
UAA
rneΔ1018::bla
XmnI
XmnI
500 bp

Figure 2.2. Schematic of the various truncated RNase E genes. Shown at the top is the gene with the catalytic region, ARRBS (arginine-rich RNA binding site), and the scaffold region for RhlB, enolase, and PNPase binding indicated. The *rneΔ225* and *rneΔ374* truncations remove 217 and 373 amino acids, respectively, from the C-terminus. The *rneΔ91* mutation results in an in-frame deletion of the ARRBS (residues 597-689). The *rneΔ508* mutation results in a C-terminus deletion of 508 amino acids. The *rneΔ610* truncation is an internal in-frame deletion of 609 residues, leaving 427 amino acids at the N-terminus (N) and the last 25 amino acids at the C-terminus (C). The two shortest truncations, *rneΔ831* and *rneΔ1034* consist of only about 230 and 27 amino acid residues, respectively, of the N-terminus. The asterisk at the C-terminus of *rneΔ225*, *rneΔ508*, *rneΔ831*, and *rneΔ1034* indicates the absence of the normal UAA translation stop codon.

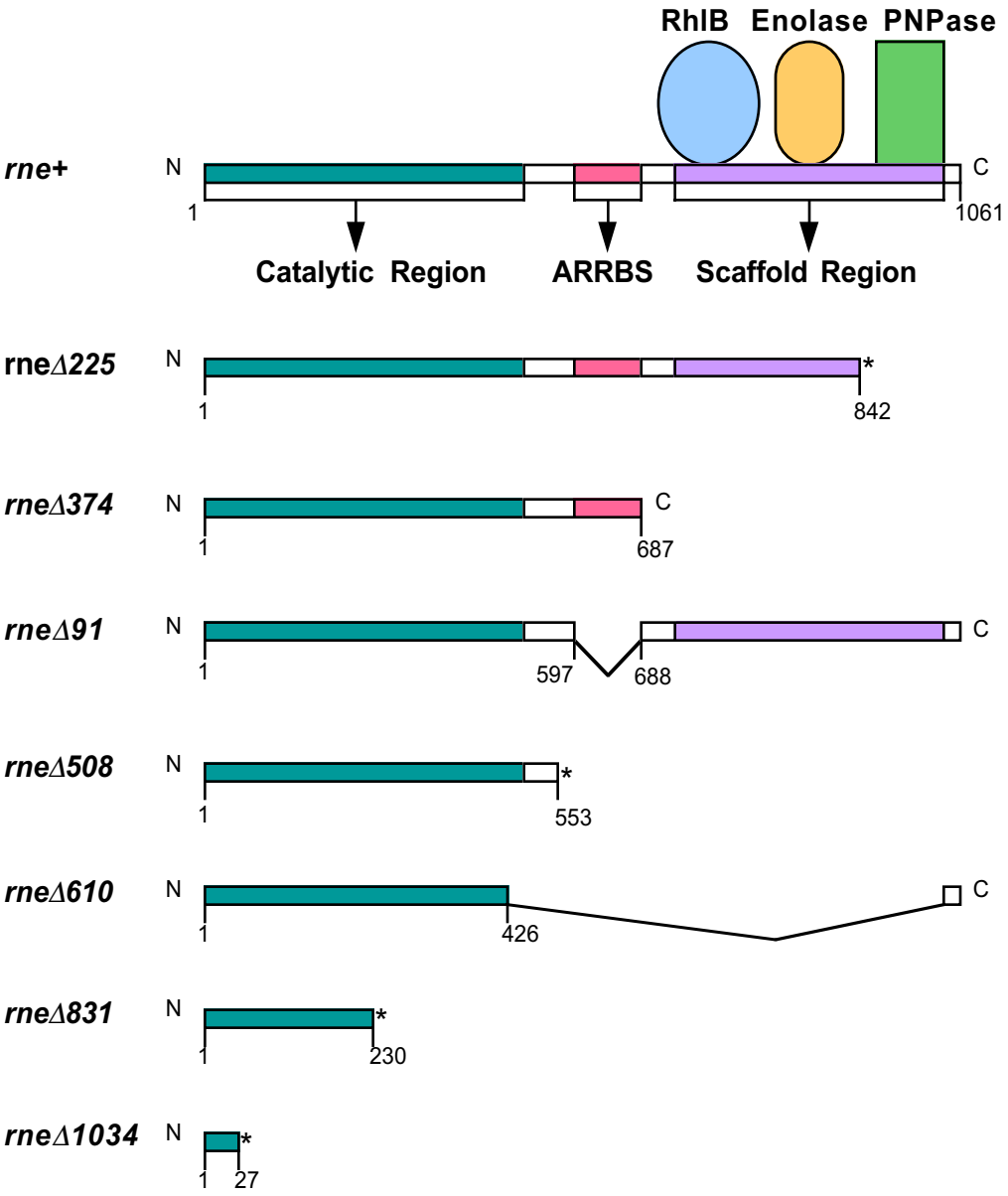

RhlB
Enolase
PNPase
rne+
N
C
1
1061
Catalytic Region
ARRBS
Scaffold Region
rneΔ225
*
842
rneΔ374
687
rneΔ91
597
688
rneΔ508
553
rneΔ610
426
rneΔ831
230
rneΔ1034
27

Figure 2.3. Growth properties of strains encoding truncated RNase E protein. All the strains were freshly streaked onto L agar plates containing 50 μg/ml of thymine and 50 μg/ml of ampicillin and incubated overnight at 37°C or 44°C. The order of the strains on both plates is described by the diagram at the top.

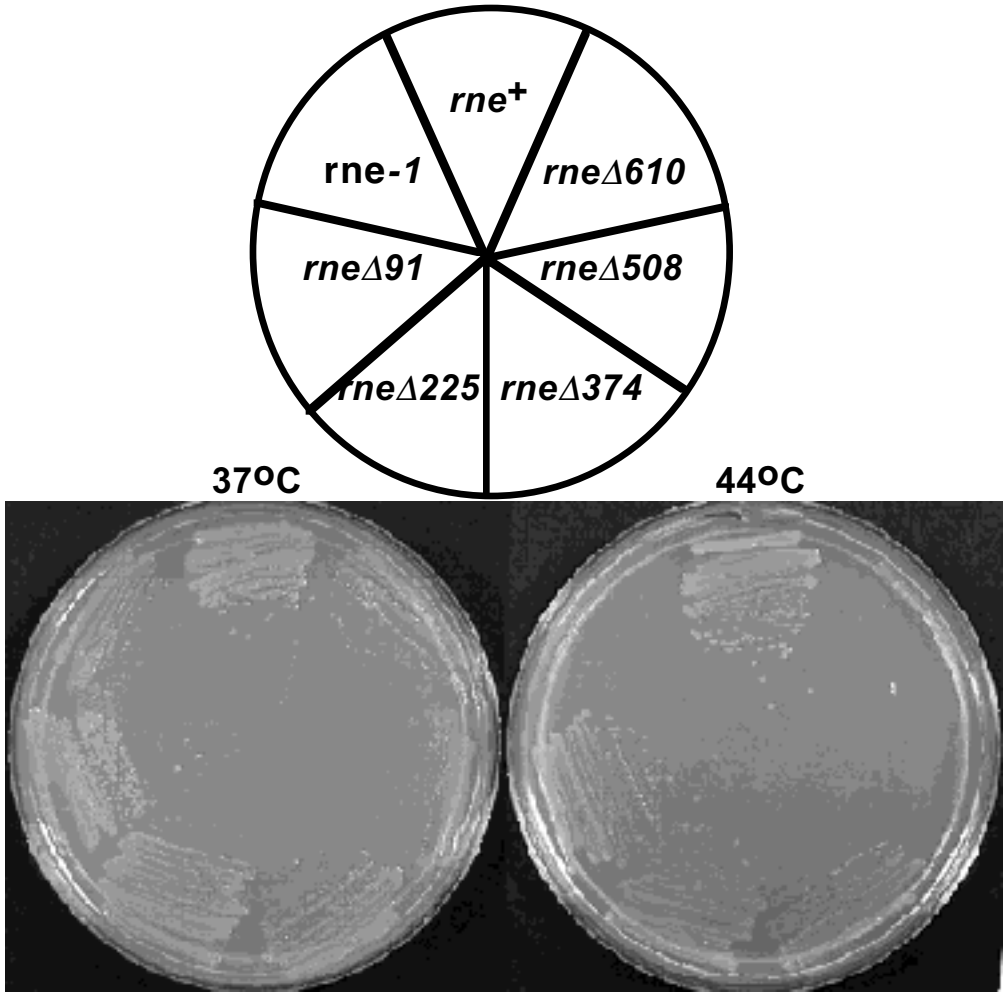

rne+
rne-1
rneΔ610
rneΔ91
rneΔ508
rneΔ225
rneΔ374
37ºC
44ºC

Figure 2.4. Immunoprecipitation of degradosome complexes from strains carrying the various truncated RNase E proteins using PNPase antibody. The top panel is a PNPase Western blot of the PNPase immunoprecipitation. The center panel is an RNase E Western done from a stripped blot of the PNPase Western and reprobed with an RNase E MAP antiserum (against the first 20 amino acids of RNase E). The last panel is a RhlB helicase Western done by stripping the RNase E Western blot and reprobing it with an RhlB helicase antiserum. Stripping of all Western blots was done as suggested by the ECL Western blotting analysis system (Amersham Life Science) by gently agitating the blot in ECL stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris pH 6.7) at 50^{o}C for 30 min. L, pi, and IP indicate total protein lysate, pre-immune serum, and immunoprecipitation, respectively. Lanes 1, 5, 9, 13, and 17 each contain 50 μg of total protein extract. Lanes 2, 3, 6, 7, 10, 11, 18, and 19 are the negative controls using pre-immune sera from two different rabbits. Lanes 4, 8, 12, 16, and 20 show the composition of the immunocomplexes in the various strains.

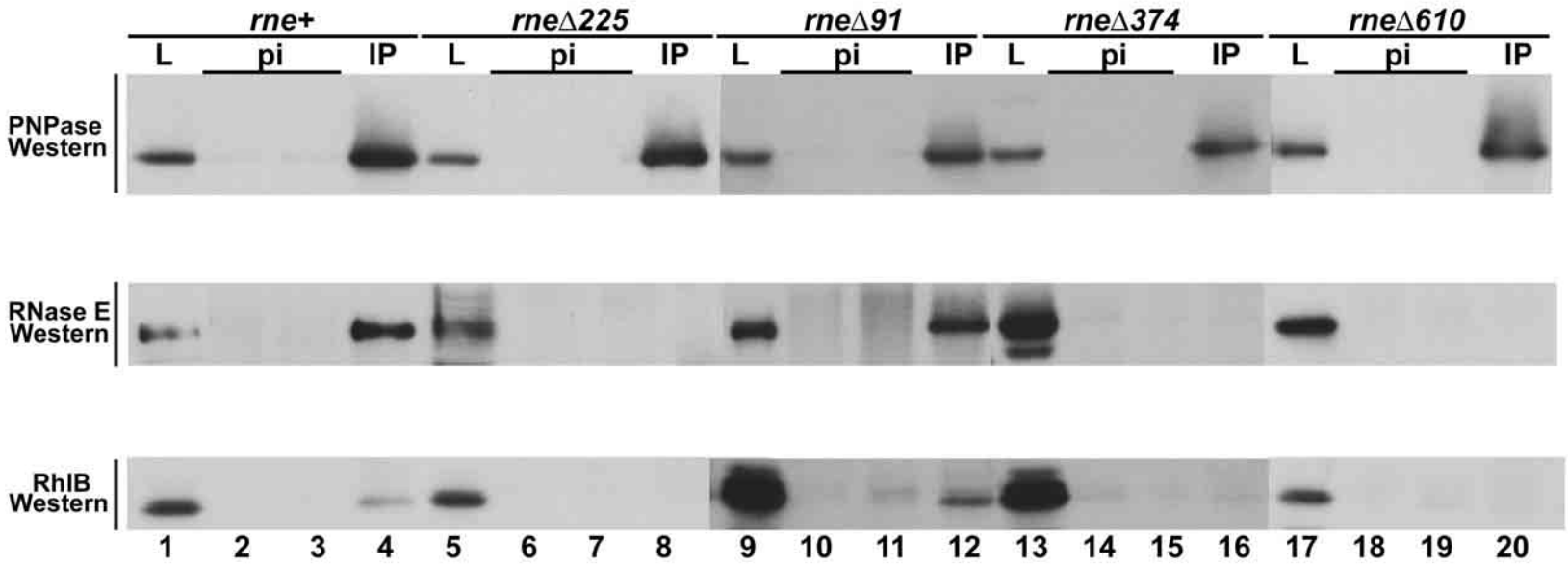

rne+
rneΔ225
rneΔ91
rneΔ374
rneΔ610
L
pi
IP
PNPase Western
RNase E Western
RhlB Western
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Figure 2.5. Northern blot analysis of the *rpsT* and *rpsO* transcripts. Total RNA was extracted from cultures growing exponentially at 37°C in rich medium as described in Experimental Procedures. An equal amount (5 μg) of RNA was loaded into each lane of a 6% polyacrylamide/7M urea. Top panel: *rpsT* decay profile. Time points 0, 1, 2, 5, 10, 20, and 30 indicate the time in minutes after rifampicin was added to stop new transcription initiation. The two arrows indicate the full-length *rpsT* transcripts generated from its two promoters. The origin of the intense third band seen in the *rneΔ610* strain is not known at this time. Bottom panel: *rpsO* decay profile. The time points are the same as in the *rpsT* Northern. The small and big arrows in the *rpsO* Northern indicate *rpsO1* (P1-RIII) and *rpsO2* (P1-t1), respectively (Hajnsdorf *et al*., 1996).

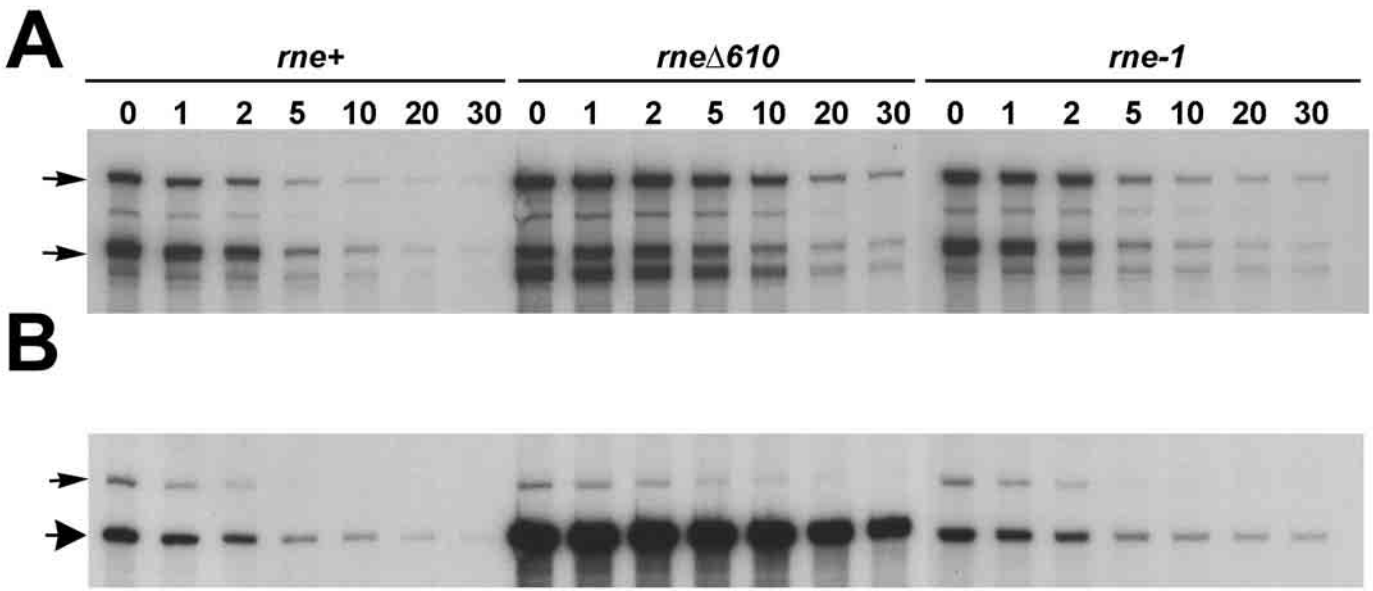

A
rne+
rneΔ610
rne-1
0 1 2 5 10 20 30 0 1 2 5 10 20 30 0 1 2 5 10 20 30
B

Figure 2.6. Western blot analysis of various RNase E proteins. Equivalent amounts of each total protein extract (50 μg/lane) were boiled for 5 min and loaded onto an 8% SDS/PAGE. A Coomassie-stained gel was also done in parallel to confirm that an equal amount of lysate was loaded into each lane (data not shown). The RNase E proteins were detected using the ECL Western blotting analysis system (Amersham Life Science) according to the specifications of the manufacturer using an RNase E MAP antibody (as described in Fig. 2.4) at a dilution of 1:2,000. Asterisks indicate the expected RNase E proteins while smaller species may be breakdown products of the larger RNase E protein. Protein size standards are indicated on the left. Lane 1, MG1693 (wild-type); lane 2, SK9714 (*rneΔ1018::bla* [pSBK1 *rne*$^+$ Cm^r]), lane 3, SK9937 (*rneΔ1018::bla* [pMOK13 *rne-1* Cm^r]); lane 4, SK9976 (*rneΔ1018::bla* [pMOK17 *rneΔ91* Cm^r]); lane 5, SK9950 (*rneΔ1018::bla* [pFMK33 *rneΔ225* Km^r]); lane 6, SK9971 (*rneΔ1018::bla* [pMOK16 *rneΔ374* Km^r]); lane 7, SK9952 (*rneΔ1018::bla* [pSYK1 *rneΔ508* Km^r]); lane 8, SK9957 (*rneΔ1018::bla* [pMOK15 *rneΔ610* Cm^r]).

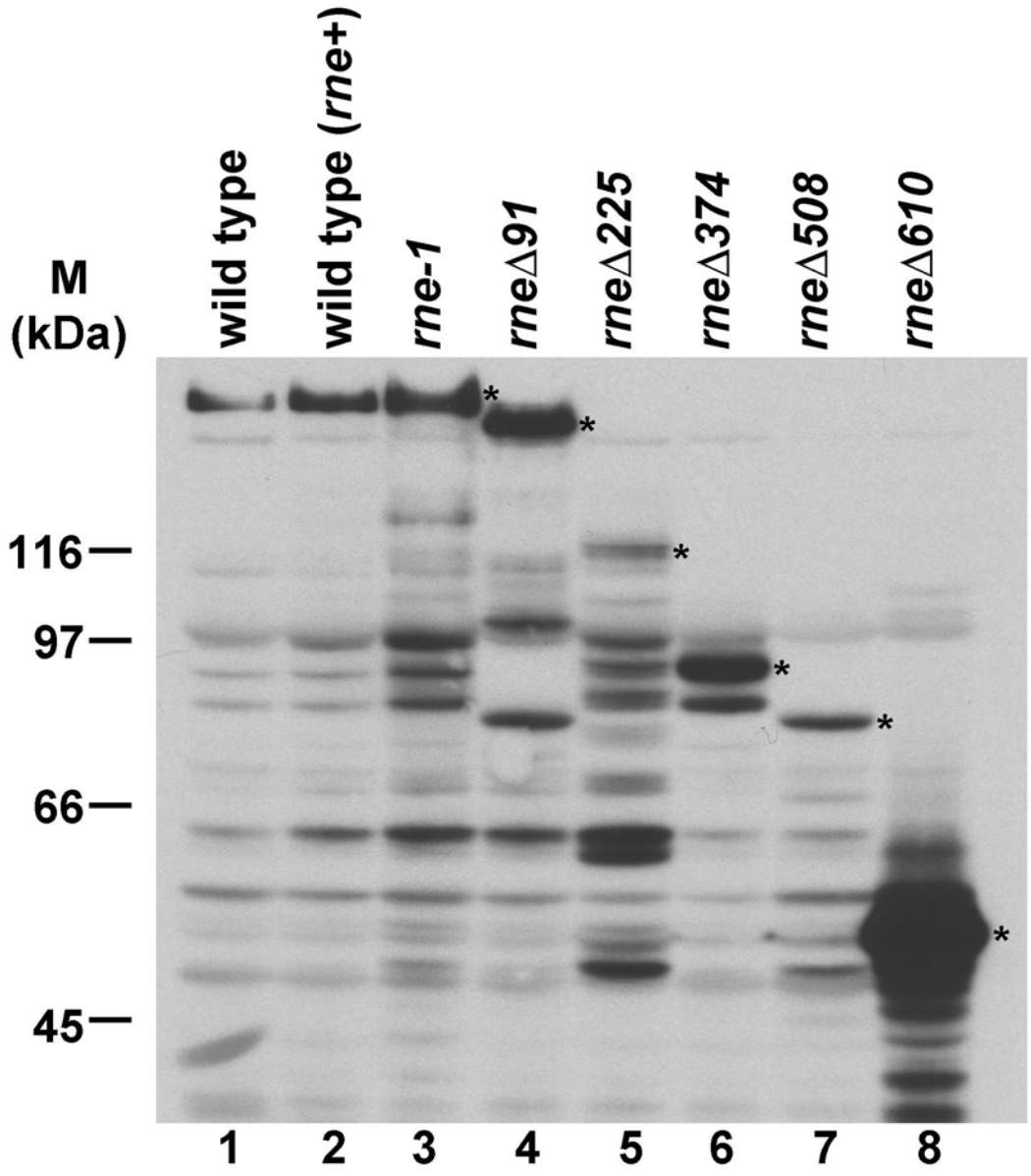

M
(kDa)
wild type
wild type (rne+)
rne-1
rneΔ91
rneΔ225
rneΔ374
rneΔ508
rneΔ610
116
97
66
45
1
2
3
4
5
6
7
8

Figure 2.7. Primer extension analysis of the *rne* transcript. Total steady-state RNA was isolated at 37°C and 50 μg (each lane) was subjected to primer extension analysis of the *rne* 5' UTR using primer QLrneL2. The small arrow points at the transcriptional start site of *rne*. The big arrow shows the RNase E cleavage sites. A sequencing ladder made from QLrneL2 is shown to the left of the primer extensions. Similar primer extension results were obtained using primer RNE+240 (data not shown). Lane 1, *rne*$^{+}$; lane 2, *rne-1*; lane 3, *rneΔ91*; lane 4, *rneΔ225*; lane 5, *rneΔ374*; lane 6, *rneΔ508*; lane 7, *rneΔ610*.

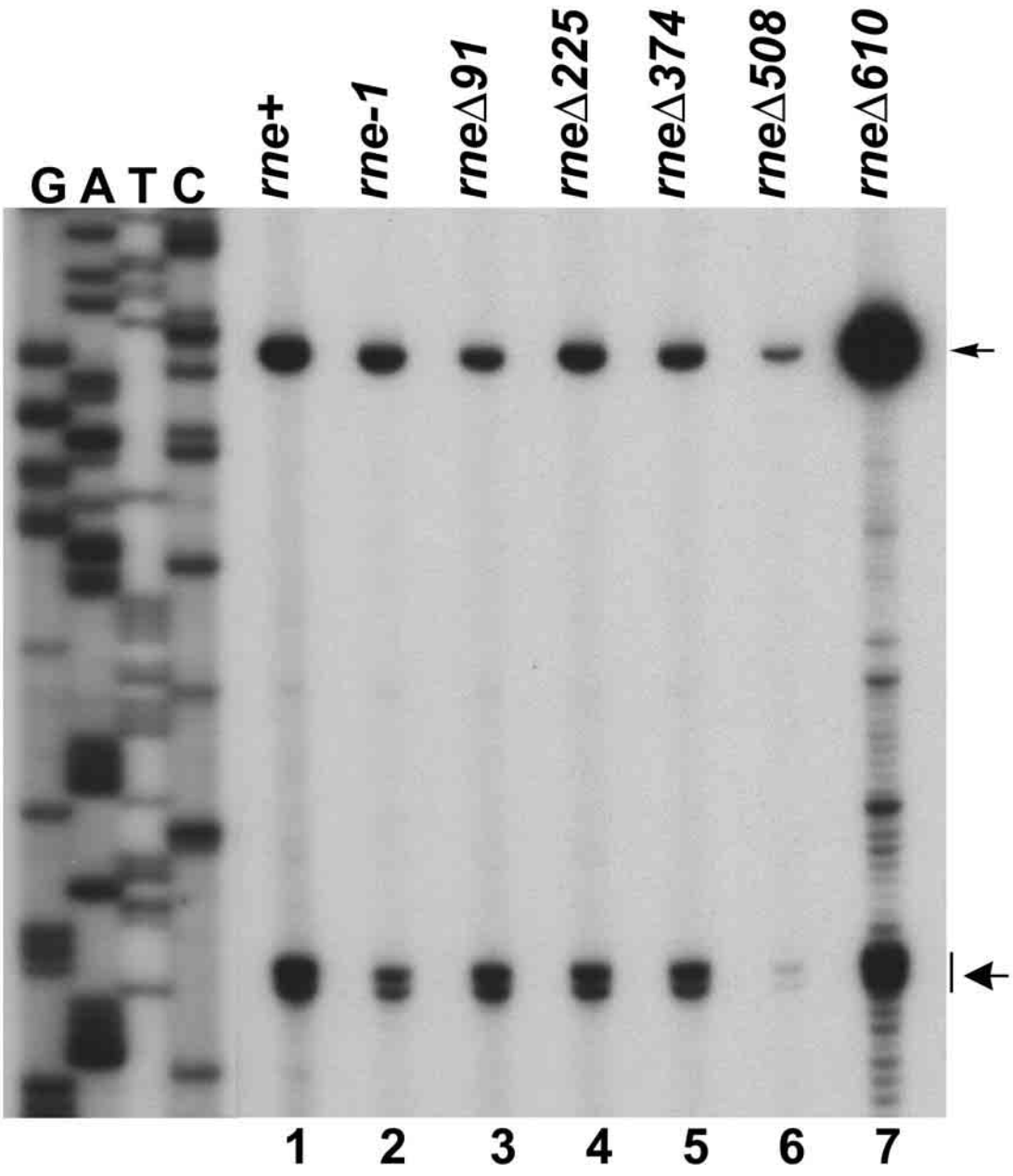
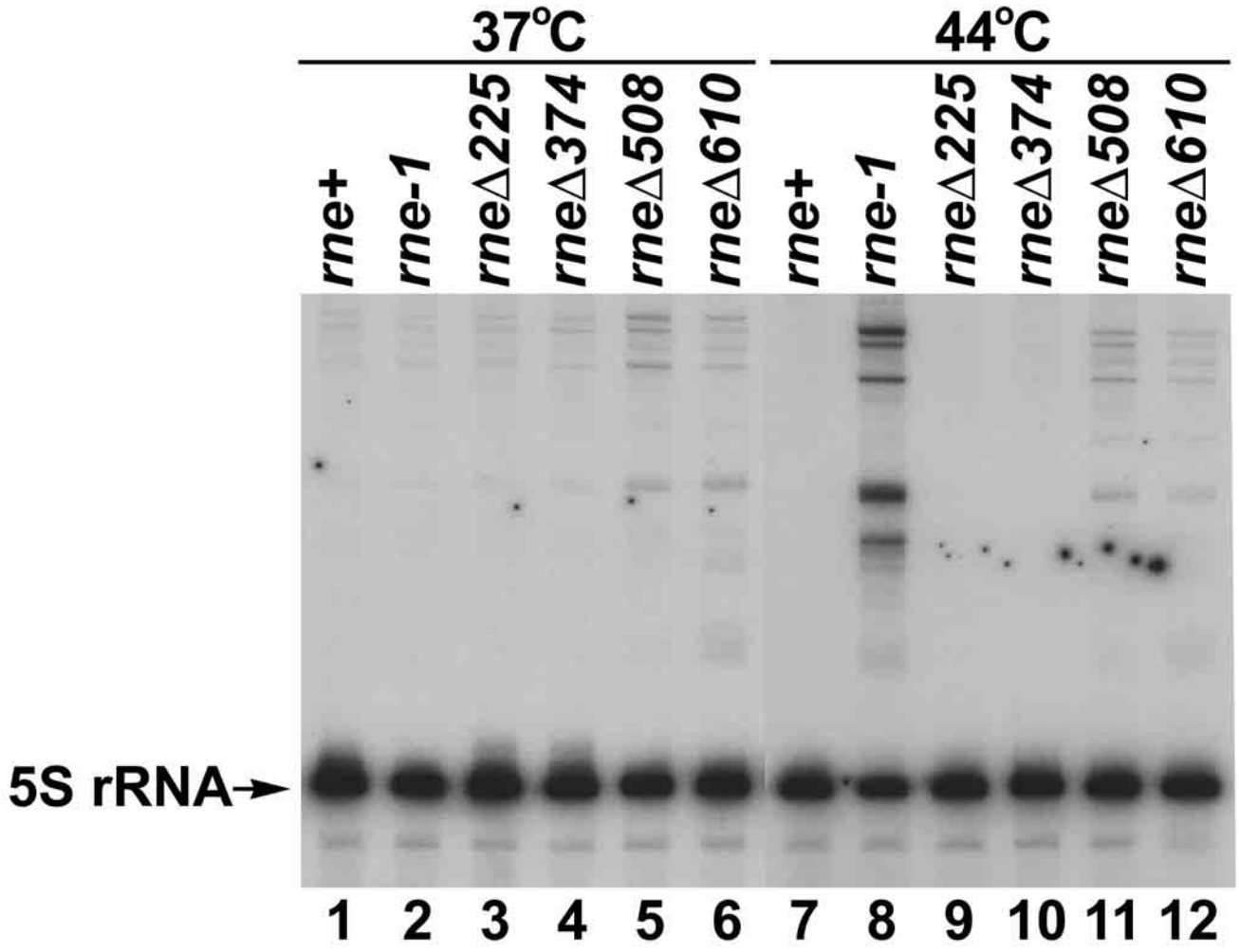

G A T C
rne+
rne-1
rneΔ91
rneΔ225
rneΔ374
rneΔ508
rneΔ610
1
2
3
4
5
6
7

Figure 2.8. *In vivo* processing of 9S rRNA by the various RNase E proteins. Total RNA was obtained from an exponentially growing culture at 37ºC in L broth (supplemented with 50 μg/ml thymine and 20 μg/ml chloramphenicol or 50 μg/ml kanamycin) as indicated in Experimental Procedures except for the omission of rifampicin and nalidixic acid. Five μg of RNA was electrophoresed in each lane of a 6% PAGE/7 M urea, electrotransferred to an MSI MagnaCharge nylon membrane (Micron Separations) and probed with a 5' end-labeled oligonucleotide (PB5SRNA, 5' CGCTACGGCGTTTCACTTCT 3') specific for the 5S rRNA (lanes 1-6). 44ºC steady-state RNA was obtained by shifting a 37ºC exponentially growing culture to 44ºC for 20 minutes (lanes 7-12). The arrow shows the mature 5S rRNA while the bands above it are processing intermediates. Lanes 1, 7, *rne*$^{+}$; lanes 2, 8, *rne-1*; lanes 3, 9, *rneΔ225*; lanes 4, 10, *rneΔ374*; lanes 5, 11, *rneΔ508*; lanes 6, 12, *rneΔ610*.


37°C
44°C
rne+
rne-1
rneΔ225
rneΔ374
rneΔ508
rneΔ610
rne+
rne-1
rneΔ225
rneΔ374
rneΔ508
rneΔ610
5S rRNA
1 2 3 4 5 6 7 8 9 10 11 12

CHAPTER 3

MATURATION OF *ESCHERICHIA COLI* tRNAs BY RNASE P AND 3' → 5' EXORIBONUCLEASES IS DEPENDENT ON RNASE E PROCESSING[1]

---

[1]Ow, M. C and S. R. Kushner. Submitted to Genes and Development, 7/30/01.

ABSTRACT

RNase E, an essential endoribonuclease in *Escherichia coli*, is known to be involved in both 9S rRNA processing and the degradation of many mRNAs. However, recent experiments have suggested that defects in 9S rRNA processing and mRNA decay are not responsible for the lack of cell growth in the absence of RNase E. Using several new *rne* mutations, we demonstrate here that the essential *in vivo* role of RNase E is the processing of tRNA transcripts. RNase E catalytic activity is required for initiating the processing of both polycistronic operons, such as *glyW cysT leuZ*, *argX hisR leuT proM*, and *tyrT tyrV tpr* and the monocistronic *pheU* transcript. Specifically, cleavage by RNase E within the immature 3' terminal region of a tRNA transcript must occur before RNase P can cleave to generate the mature 5' terminus and the various 3' → 5' exonucleases can function to produce the mature 3' CCA terminus. Taken together with earlier work, these data show that RNase E is intimately involved in the processing and decay of every major type of RNA species in the bacterium.

INTRODUCTION

Ribonuclease E (RNase E) of *E. coli* was first characterized in a temperature-sensitive mutant (*rne-3071*) that accumulated 9S precursors of the 5S rRNA at 42°C (Ghora and Apirion, 1978). Independently, the *ams* (*a*ltered *m*RNA *s*tability) gene was identified because of its ability to affect the decay of total pulse-labeled RNA at elevated temperatures (Ono and Kuwano, 1979). Subsequently, both loci were shown to encode RNase E (Mudd *et al*., 1990; Babitzke and Kushner, 1991; Taraseviciene *et al*., 1991). The *rne* gene encodes a 1061 amino acid (aa) protein (Casarégola *et al*., 1992; 1994) that has now been characterized as a 5'-end-dependent endoribonuclease (Mackie, 1998). In addition, it has also been demonstrated that RNase E is part of a multiprotein complex, called the degradosome that includes polynucleotide phosphorylase (PNPase), the RhlB

RNA helicase and the glycolytic enzyme enolase (Carpousis *et al*., 1994; Py *et al*., 1994; 1996; Miczak *et al*., 1996).

In vivo experiments with either the *rne-1* or *rne-3071* thermolabile alleles demonstrated the accumulation of unprocessed 5S rRNA intermediates (Ghora and Apirion, 1978) and a general slowing in the decay of specific mRNA transcripts (Arraiano *et al*., 1988; Mackie, 1991; Régnier and Hajnsdorf, 1991). As such it was assumed that the inviability associated with the inactivation of RNase E resulted from either a defect in 9S rRNA processing or mRNA decay. However, experiments by López *et al*. (1999) and Ow *et al*. (2000) suggest that this hypothesis cannot explain the inviability of *rne* temperature-sensitive mutants. Using *rne* truncation mutations, both laboratories showed that 9S rRNA processing was normal under conditions where mRNA decay was significantly impaired (López *et al*., 1999; Ow *et al*., 2000). Furthermore, Ow *et al*. (2000) characterized an extensive RNase E C-terminus deletion mutation (*rneΔ610*) that was missing 609 aa which included the ARRBS (arginine-rich-RNA binding site) and the degradosome scaffolding region (Fig. 3.1). This protein was able to support cell viability at 37ºC but not at 44ºC. More importantly, mRNA decay was more defective at 37ºC than in a *rne-1* mutant shifted to the nonpermissive temperature. Even at 44ºC, the nonpermissive temperature for the *rneΔ610* strain, 9S rRNA processing was normal. It was thus concluded that inviability in the absence of RNase E was not associated with defects in either mRNA decay or 9S rRNA processing (Ow *et al*., 2000).

Since these experiments appeared to rule out both mRNA decay and 9S rRNA processing as the essential function of RNase E, we sought to determine what other aspect of RNA metabolism required the activity of this enzyme. A possible candidate was tRNA processing. Although 14 tRNAs occur within the seven rRNA transcription units, 72 are found as part of independent transcription units, including some that are polycistronic (Berlyn, 1998). While these transcripts could be processed by a combination of an endonucleotlytic cleavage by RNase P at the 5' end (Altman *et al*., 1995) and exonucleolytic degradation at the 3' end by RNase II, RNase BN, RNase PH, RNase D, RNase T, and

PNPase (Li and Deutscher, 1996), it was also possible that RNase E might be required to cleave within the intercistronic regions. Failure to process tRNAs properly would lead to a cessation of protein synthesis and concomitantly cell growth. In fact, when Ray and Apirion (1981a) isolated small RNAs from a *rne-3071* mutant shifted to 42ºC, they found 5S rRNA precursors as well as molecules that contained both $tRNA^{Leu}$ and $tRNA^{His}$. These two tRNAs are part of a four gene (all tRNAs) transcription unit, *argX hisR leuT proM* (Berlyn, 1998). Subsequently, they showed that if such tRNA precursors were treated *in vitro* with RNase E, then RNase P (the endoribonuclease responsible for generating the mature 5' end of tRNAs) could cleave at the 5' termini (Ray and Apirion, 1981b).

Accordingly, we sought to examine the effect of inactivating RNase E on the generation of mature tRNAs. In this communication, we report the analysis of tRNA processing employing a variety of RNase E mutant strains. Of particular interest was the fortuitous isolation of a temperature-resistant revertant of the *rneΔ610* allele (Ow *et al*., 2000). The *rneΔ645* mutation encodes an RNase E protein of only 417 aa but unlike *rneΔ610* permits cell growth at both 37ºC and 44ºC. While processing of both polycistronic and monocistronic tRNAs was impaired at 44ºC in *rne-1*, *rneΔ610*, and *rneΔ645* strains, transcripts were processed 2-3.7-fold faster in the *rneΔ645* mutant compared to the *rne-1* and *rneΔ610* strains. There was a direct correlation between tRNA processing rates, the relative amounts of mature tRNAs, and cessation of cell growth. In addition, RNase P cleavage at the 5' end of tRNA transcripts did not proceed in the absence of RNase E cleavage at the 3' end. Furthermore, inactivation of several of the 3' → 5' exonucleases involved in the generation of the mature 3' CCA terminus (RNase II, RNase D, RNase BN, and RNase T) (Li and Deutscher, 1996) did not affect either RNase E or RNase P processing.

RESULTS

Isolation and characterization of a temperature-resistant revertant of the *rneΔ610* allele, *rneΔ645*

It has recently been shown that the product of the *rng* gene encodes a ribonuclease, called RNase G, that is involved in the maturation of the 5' end of the 16S rRNA (Li *et al*., 1999; Wachi *et al*., 1999). More importantly, the amino acid sequence of the N-terminus of RNase E is 34% identical to the RNase G protein, and there appears to be some functional relationship between the two proteins (McDowall *et al*., 1993; Wachi *et al*., 1997). This raised the possibility that RNase G might also be involved in mRNA decay. To test this hypothesis, we constructed a strain, SK9982 (*rneΔ1018::bla rng::cat thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pMOK19 [*rneΔ610* Sm^r]), carrying mutations in both RNase E and RNase G. The mRNA decay properties of this strain will be reported elsewhere (Ow, M.C. and S. R. Kushner, manuscript in preparation).

During our initial characterization of the growth properties of SK9982, we replica-plated the strain onto Luria agar plates that were subsequently incubated at 44°C. While the cell patches for SK9982 initially did not grow at the elevated temperature, we noticed the appearance of small individual colonies within the replica patches that eventually grew into large colonies. In contrast, the negative control, SK9957 (*rneΔ1018::bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pMOK15 [*rneΔ610* Cm^r]), exhibited no growth (data not shown). We picked eight individual colonies for further study. After purification, all eight grew at 44°C, in contrast to the SK9957 (*rneΔ610*) control.

Plasmid DNA was isolated from each of the eight temperature-resistant revertants and was used to displace pSBK1 (*rne*$^+$ Cm^r) in SK9714 (*rneΔ1018::bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pSBK1[*rne*$^+$ Cm^r]). Transformants derived from seven of the eight strains had properties that were identical to SK9957 (*rneΔ1018::bla*/ *rneΔ610*), *i.e*., they grew at 37°C but not at 44°C. This result suggested the existence of second site chromosomal suppressors in the strains from which these plasmids were isolated.

However, one of the revertants contained a plasmid, which we named pMOK21, that led to temperature-resistant growth in the *rneΔ1018::bla* genetic background. Since we could easily characterize the *rne* allele carried on pMOK21 by DNA sequencing, we chose this strain, which we named SK9987 (*rneΔ1018::bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pMOK21 [revertant of *rneΔ610* Sm^r]), for further characterization.

DNA sequencing of the complete *rne* gene in pMOK21 revealed a single nucleotide change, a C to A transversion within the codon for serine 418, resulting in a stop codon (Fig. 3.1). This new premature stop codon further shortened the protein from the RneΔ610 parent by 35 amino acids, generating a polypeptide, RneΔ645, of 417 amino acids that migrated as ~46 kDa protein in an 8% SDS polyacrylamide gel (Figs. 3.1, 3.2). As has previously been observed with RneΔ610 (Ow *et al*., 2000), there appeared to be a complete loss of autoregulation (*i.e*., a significant increase in RneΔ645 protein level as compared to the level of wild-type RNase E protein, Fig. 3.2). To confirm that the C to A transversion was responsible for the suppression of lethality observed in SK9987, we used site-directed mutagenesis to reconstruct the *rneΔ645* mutation in pMOK15 (*rneΔ610* Cm^r) creating pMOK29 (*rneΔ645* Cm^r). Transformation of pMOK29 into SK9714 and the subsequent displacement of the resident plasmid (pSBK1, *rne*$^+$) resulted in a strain, SK10103 (*rneΔ1018::bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pMOK29 [*rneΔ645* Cm^r]), whose properties were identical to SK9987.

Growth properties of the *rneΔ645* mutant

On Luria agar medium, SK9987 (*rneΔ645*) took two days at 44ºC to form colonies compared to one day for the wild-type control (SK9714). In Luria broth, SK9987 continued to grow at 44ºC (30 min generation time), albeit slightly slower than the wild-type SK9714 (26 min) control (Fig. 3.3). In contrast, SK9957, the *rneΔ610* progenitor, ceased growing within 4 hrs after shift to the nonpermissive temperature (Fig. 3.3). A control strain, SK9937 (*rneΔ1018::bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pMOK13 [*rne-1*

Cm^r]) harboring the *rne-1* temperature-sensitive allele ceased growing between 60-120 min after the shift to 44°C (Fig. 3.3).

mRNA decay is still significantly impaired in the *rneΔ645* mutant

Based on our previous analysis of the *rneΔ610* allele, we had hypothesized that the conditional lethality associated with *rne* mutations was not related to defects in mRNA decay (Ow *et al*., 2000). If this idea were correct, we predicted that mRNA decay in the *rneΔ645* strain should still be very defective. Accordingly, we examined the decay of two RNase E-dependent transcripts, *rpsT* and *rpsO* (Mackie, 1991; Hajnsdorf *et al*., 1994). The *rpsO* and *rpsT* mRNAs decayed between 1.5-1.7-fold more rapidly in SK9987 (*rneΔ645*) than in SK9957 (*rneΔ610*), but their decay rates were still much slower than that of the wild-type and *rne-1* strains (Table 3.1). In fact, their half-lives in the *rneΔ645* strain at 37°C were still equal to or longer than those observed in a *rne-1* mutant at the nonpermissive temperature (Ow *et al*., 2000).

9S rRNA processing in the *rneΔ645* revertant

Ghora and Apririon (1978) first identified the *rne* gene because of its affect on the processing of a 9S rRNA precursor into a p5S rRNA species. Because of our previous observation that 9S rRNA processing was relatively normal in the *rneΔ610* mutant (Ow *et al*., 2000), we did not expect to see any significant differences between the *rneΔ610* and *rneΔ645* strains. To test this hypothesis, we performed a Northern blot using a 5S rRNA probe on total RNA isolated from wild-type, *rne-1, rneΔ610*, and *rneΔ645* strains that had been grown at 37°C and subsequently been shifted to 44°C. As expected, in the *rne-1* strain, larger rRNA precursors accumulated when the strain was shifted from 37°C to 44°C (Fig. 3.4, lanes 2, 6). In contrast, only a few precursors molecules were observed in a wild-type control (Fig. 3.4, lanes 1, 5). As previously reported (Ow *et al*., 2000), with the *rneΔ610* mutant some precursors were observed at 37°C, but most importantly, upon shift to 44°C the amount of precursors did not increase (Fig. 3.4, lanes 3, 7). A similar result

was obtained in the *rneΔ645* strain (Fig. 3.4, lanes 4, 8). Overall, 9S rRNA processing appeared slightly more efficient in the *rneΔ645* strain compared to the *rneΔ610* mutant.

Processing of polycistronic transcripts containing tRNAs requires functional RNase E activity

Since the changes in mRNA decay rates and 9S rRNA processing did not seem sufficient to account for the ability of the *rneΔ645* strain to grow at 44°C, we sought to determine what other aspect of RNA metabolism might have been affected. Because of previous observations by Ray and Apirion (1981a; 1981b) that the RNA species that accumulated at the nonpermissive temperature in cells encoding the conditional lethal *rne-3071* allele contained $tRNA^{Leu}$ and $tRNA^{His}$, we decided to examine tRNA processing in rne^{+}, *rne-1*, *rneΔ610*, and *rneΔ645* strains at 44°C. If RNase E were involved in tRNA processing, we assumed that its most likely role would be in the processing of polycistronic operons that contained several tRNAs. In addition, we hypothesized that we might see the most dramatic effect with tRNAs for which there was only a single copy of the gene. Accordingly, we chose to examine the *glyW cysT leuZ* and *argX hisR leuT proM* polycistronic transcripts (Figs. 3.5a, 3.6a) because the *cysT* and *hisR* genes are unique and because these operons contained reasonably large spacer regions between the tRNAs.

Using oligonucleotides complementary to the 3' spacer region between the *cysT* and *leuZ* and to the 3' spacer region between the *hisR* and *leuT* tRNA genes (cysT-leuZ and hisR-leuT, Table 3.5, Figs. 3.5a, 3.6a), we performed Northern analyses employing steady-state RNA isolated from cells that had been shifted to 44°C to determine whether processing intermediates could be detected in strains carrying the rne^{+}, *rne-1*, *rneΔ610*, and *rneΔ645* alleles. In the initial experiment, we only observed the mature tRNAs in the wild-type control but could see a variety of processing intermediates in all three mutant strains (data not shown). Since there were unprocessed tRNA precursors in all three RNase E mutants, we decided to measure the decay rates of the large transcripts to determine if there were significant differences among the strains at 44°C. In particular, we wanted to ascertain if

tRNA precursors were processed more efficiently in the *rneΔ645* strain compared to either *rneΔ610* or *rne-1*.

Half-life experiments were carried out using a probe (cysT-leuZ, Table 3.5) for the intercistronic region between the *cysT* and *leuZ* genes (Figs. 3.5a, 3.5b). While no precursor transcripts were visible in the wild-type control, two large species of 460 (full length) and 290 nucleotides (nt) were observed in the *rne-1*, *rneΔ610*, and *rneΔ645* mutants along with a series of smaller intermediates ranging in size from 90-200 nt (Fig. 3.5b). Half-life determinations for the largest species showed that the *rneΔ645* strain processed it 2-fold faster than the *rneΔ610* mutant and 2.8-fold faster than the *rne-1* strain (Table 3.2). In fact, both the *rneΔ610* and *rneΔ645* strains processed this transcript significantly faster than the *rne-1* mutant (Table 3.2). As a control, the membrane was stripped and reprobed for the mature $tRNA^{Cys}$ (Fig. 3.5d). Quantitation of the amount of mature $tRNA^{Cys}$ correlated with the growth properties observed in Table 3.3. Specifically, the wild-type strain contained the most mature $tRNA^{Cys}$, followed by *rneΔ645*, *rneΔ610,* and *rne-1* (Table 3.3).

To confirm that these observations were not unique to the *glyW cysT leuZ* operon, we next tested the *argX hisR leuT proM* polycistronic transcript. As shown in Fig. 3.6b, a probe (hisR-leuT, Table 3.5, Fig. 3.6a) for the spacer region between *hisR* and *leuT* detected two large precursor transcripts of 530 (full length) and 335 nt in addition to smaller species between 85-225 nt in length in the *rne* mutants. Again no precursors were seen in the wild-type control. With this operon, the decay of the full-length precursor was almost identical in the *rne-1* and *rneΔ610* strains (Table 3.2) and much slower than that observed for the *glyW cysT leuZ* operon. However, the processing in the *rneΔ645* strain was 3.7-fold faster than in either the *rne-1* or *rneΔ610* mutants (Table 3.2). Re-probing the blot with a probe specific for the mature $tRNA^{His}$ again showed that the *rneΔ645* strain contained more than either the *rneΔ610* or *rne-1* mutants (Fig. 3.6d, Table 3.3).

We next examined if RNase E was also involved in the processing of tRNA operons that contained multiple copies of specific tRNAs. For these experiments we chose

the *tyrT tyrV tpr* (Fig. 3.7a) and *lysT valT lysW valZ lysY lysZ lysQ* (data not shown) operons. Interestingly, when Northerns were probed with an oligonucleotide (tyrSP, Table 3.5) that would hybridize to the *tyrT tyrV* spacer region (Fig. 3.7a), the decay rates for the largest precursors were almost identical among the three *rne* mutants (Table 3.2; Fig. 3.7b). However, there clearly was more mature $tRNA_1^{Tyr}$ in the *rneΔ645* strain compared to the *rne-1* strain (Fig. 3.7d, Table 3.3). Immature tRNA intermediates were also detected with the *lysT valT lysW valZ lysY lysZ lysQ* polycistronic operon using primer lysT-valT (Table 3.5). Their decay rates also appeared to be comparable among the three *rne* mutants (data not shown).

Processing of monocistronic tRNA transcripts also requires functional RNase E activity

Since RNase E processing within the spacer regions between tRNAs in polycistronic transcripts appeared to be absolutely essential, we also wanted to determine if the enzyme was also required for the processing of monocistronic tRNAs. As a test transcript, we chose *pheU* because it contains approximately 44 nt downstream of the mature CCA terminus (Fig. 3.8a) and is almost identical in sequence organization to *pheV*, the other phenylalanine tRNA in *E. coli*. Thus, analysis of the *pheU* transcript would allow us to simultaneously measure processing of both phenylalanine tRNAs. However, because these transcripts have relatively short 3' ends, we assumed that the various 3' → 5' exonucleases (RNase II, RNase BN, RNase PH, RNase T, and RNase D) that have been shown to be involved in the generation of mature 3' CCA termini (Li and Deutscher, 1996) would be sufficient.

To our surprise, a 120 nt long unprocessed form of $tRNA^{Phe}$ was observed in all three *rne* mutant strains (Fig. 3.8b). Of even more interest was the greater dependence on RNase E processing of the monocistronic transcript than the polycistronic transcripts (*e.g.*, for *rne-1* compare 31.8 min for *pheU* versus 9.9 min for *cysT leuZ*, 14.5 min for *hisR leuT*, and 8.9 min for *tyrT tyrV,* Table 3.2). However, as was seen for the *glyW cysT leuZ* and *argX hisR leuT proM* operons, processing was faster in the *rneΔ645* strain compared to

either the *rne-1* or *rneΔ610* mutants (Table 3.2). More importantly, processing occurred 2.3-fold faster in the *rneΔ645* strain compared to the *rneΔ610* mutant. No intermediates were detected in the *rne*$^{+}$ strain (Fig. 3.8b). As observed with the polycistronic transcripts, the level of tRNA^{Phe} was significantly reduced in the *rne-1* mutant compared to *rne*$^{+}$, *rneΔ610*, and *rneΔ645* strains (Fig. 3.8b, Table 3.3).

Maturation of the 5' terminus of tRNA precursors requires RNase E

The maturation of the 5' termini of tRNAs requires endonucleolytic cleavage by RNase P, another essential *E. coli* endoribonuclease (Altman, 1995). Previous *in vitro* experiments by Ray and Apirion (1981b) suggested that the action of RNase E might influence RNase P cleavage at the 5' terminus of an unprocessed tRNA transcript. To examine whether *in vivo* RNase P processing of the 5' terminus was affected by RNase E cleavage at the 3' end, we performed Northern analyses with probes designed to detect the presence of unprocessed 5' termini. In the first experiment, we used an oligonucleotide probe that was complementary to the 5' end of tRNA^{Cys} (glyW-cysT, Table 3.5, Fig. 3.5c). As observed with the 3' end probe, no processing intermediates were detected in the wild-type control (Figs. 3.5b, 3.5c). However, a series of unprocessed intermediates were clearly visible in the *rne-1* strain. In this case, the largest species decayed with a half-life of 5.9 min. Interestingly, the level of intermediates was much less pronounced in the *rneΔ610* strain and barely detectable in the *rneΔ645* mutant (Fig. 3.5c).

When a similar experiment was carried out using a probe for the 5' region of the *hisR* gene (argX-hisR, Table 3.5), a different picture emerged. In this case, significant amounts of the full-length transcript were seen in all three *rne* mutants (Fig. 3.6c). However, processing of this intermediate in the *rneΔ645* mutant was 3.0-3.7-fold more proficient than either the *rne-1* or *rneΔ610* strains (Table 3.2). Since both of these results could possibly be explained by the failure of RNase E to cut between the *glyW* and *cysT* tRNAs or the *argX* and *hisR* tRNAs, we also tested for 5' end processing of the *tyrT* tRNA which is the first gene in the *tyrT tyrV tpr* transcript (Fig. 3.7a). As observed with the other

operons we examined (Figs. 3.5c, 3.6c), processing of the 5' region by RNase P required RNase E cleavage between the *tyrT* and *tyrV* genes (Fig. 3.7c). Interestingly, even though the half-lives obtained using a probe for the 3' end of *tyrT* were not significantly different, processing at the 5' end occurred much more efficiently in the *rneΔ610* and *rneΔ645* strains (Table 3.2). No processing intermediates were detected in the *rne*$^{+}$ strain (Fig. 3.7c).

tRNA processing by RNase E and RNase P does not require the presence of 3' → 5' exoribonucleases

The current model for tRNA processing includes a series of 3' to 5' exoribonucleases (RNase II, D, BN, T, PH, and PNPase) that trim the 3' trailer sequence of each tRNA precursor to generate the mature 3' CCA (Deutscher, 1995). Since the results obtained from probing the 5' terminus of the precursor showed that RNase P cleavage at the 5' end required the prior action of RNase E (Fig. 3.7c), we decided to determine if there might also be a relationship between RNase E cleavage and 3' end exonucleolytic processing. In the first experiment, we performed Northern analysis on RNA isolated at 37°C from strains CA265 (wild-type) and CAN20-12E/18-11 (a strain deficient in RNases II, D, BN, and T; Deutscher *et al*., 1985). When we probed with an oligonucleotide complementary to the $tRNA_1^{Tyr}$, we only detected the 85 nt long $tRNA_1^{Tyr}$ in both strains (data not shown). Even after prolonged exposure of the autoradiograph, no processing intermediates were seen in either strain. Using oligonucleotides complementary to the 3' termini (tyrSP, Table 3.5) as well as the 5' end (tyrT5'A, Table 3.5) to reprobe the same membrane failed to detect any precursors in either strain (data not shown).

Because CAN20-12E/18-11 retained about 30% of RNase T (the most effective of the 3' to 5' exoribonucleases involved in tRNA processing) activity (Kelly *et al*., 1992; Deutscher *et al*., 1995), we wanted to determine whether the results obtained from CAN20-12E/18-11 would be similar to those using a strain, SK10148 (*Δrnt::kan rph-1*), in which the *rnt* (the RNase T gene) was deleted. Northern analysis using a primer complementary to $tRNA_1^{Tyr}$ did not yield any of the 95-295 nt long processing intermediates seen in the

various *rne* mutants (Figs. 3.7b, 3.7c). However, a slightly larger (~90 nt) precursor was observed in SK10148 (*Δrnt::kan rph-1*) but not the wild-type control (Fig. 3.9). This was expected because the total loss of RNase T and RNase PH activities results in the incomplete processing of the 3' terminus (Li and Deutscher, 1996).

DISCUSSION

In this communication, we have shown that RNase E has another critical function in *E. coli* RNA metabolism, namely the processing of tRNA transcripts. While previous *in vitro* work by Apirion and co-workers had suggested such a possible role for RNase E (Ray and Apirion 1981a; 1981b), our isolation and characterization of a temperature-resistant revertant of the *rneΔ610* truncation mutation has allowed us to demonstrate *in vivo* not only that RNase E is involved in tRNA processing but also that this is probably its essential function in the cell.

What is the evidence to support this conclusion? In the first place, even though the *rneΔ645* strain was able to grow at 44ºC (Fig. 3.3), the mRNA decay defects compared to the *rneΔ610* parent were not significantly altered at 37ºC. In fact, the half-lives of the *rpsO* and *rpsT* mRNAs were still longer at 37ºC than observed in the temperature-sensitive *rne-1* strain at the nonpermissive temperature (Ow *et al*., 2000). In addition, the processing of the 9S rRNA precursor was almost identical in both strains (Fig. 3.4). These results tended to rule out mRNA decay and 9S rRNA processing as the reason for the improved viability of the *rneΔ645* mutant.

In contrast, all three *rne* mutations tested here (*rne-1 rneΔ610*, and *rneΔ645*) demonstrated defects in the processing of both polycistronic (Figs. 3.5-3.7) and monocistronic (Fig. 3.8) tRNA transcripts. In fact, there was a direct correlation between the ability to grow at 44ºC, the defects in tRNA processing and the relative amounts of the mature tRNAs. Thus, as shown in Fig. 3.3, the *rne-1* strain ceased growing between 1-2 hrs after shift to 44ºC, while it took the *rneΔ610* mutant between 3-4 hrs to stop growing. On the other hand, the *rneΔ645* strain continued to grow although with a longer generation

time than the wild-type control. If one now compares the half-lives of the precursor tRNA transcripts (Table 3.2), the processing reactions catalyzed by RNase E were most impaired in the *rne-1* strain. In addition, processing with three of the four substrates tested (Table 3.2) occurred between 2.0-3.7-fold faster in the *rneΔ645* strain compared to the *rneΔ610* parent. Furthermore, there were always higher levels of mature tRNAs in the *rneΔ645* mutant compared to the *rne-1* and *rneΔ610* strains (Table 3.3). Thus, one would expect the *rne-1* strain to experience a shortage of tRNAs at 44ºC far more quickly than the *rneΔ610* or *rneΔ645* strains leading to the more rapid cessation of cell growth that was observed (Fig. 3.3).

Our data also suggest that RNase E may be required for the processing of all tRNAs or at least those that are not contained within the rRNA operons (14 tRNAs). We specifically looked at 18 tRNAs which account for 25% of the remaining 72 tRNAs in the bacterium. None of them were processed properly in all three *rne* mutants (Figs. 3.5-3.8). Of particular interest was the failure to observe *pheU* processing even though it would have seemed likely that the multiple 3' to 5' exonucleases involved in 3' end maturation (Deutscher, 1995) would have been sufficient to complete this task. It thus seems likely that the exonucleases are only capable of removing a very limited number of nucleotides from the 3' ends of tRNA transcripts after RNase E cleavage has taken place.

The data presented here confirm the earlier *in vitro* observations of Ray and Apirion (1981b). They showed that if tRNAs precursors isolated from *rne-3071* shifted to 42ºC were treated with an RNase E containing extract, RNase P could cleave the tRNA to generate a mature 5' end. As shown in Figs. 3.5-3.7, RNase E cleavage *in vivo* was a necessary prerequisite for RNase P cleavage. In addition, it was the action of RNase E on a tRNA precursor that was critical for RNase P cleavage, not the final maturation of the CCA 3' terminus by exonucleolytic processing (Fig. 3.9). Thus it seems likely that the major reason why RNase E mutants are inviable is because RNase P processing of the 5' termini and exonucleolytic processing of the 3’ end of tRNAs are blocked.

A number of other interesting questions are raised by the data reported here. One relates to the recognition of various substrates by RNase E. In all three *rne* mutants (*rne-1*, *rneΔ610,* and *rneΔ645*), tRNA processing was significantly impaired compared to the wild-type control. The main difference among the three strains was the speed with which they processed the precursor transcripts and the steady-state levels of the mature tRNAs. However, even though both the *rne-1* and *rneΔ610* strains stopped growing at 44ºC, 9S rRNA processing was almost normal in the *rneΔ610* mutant (Fig. 3.4). In contrast, the decay of mRNAs in the *rneΔ610* and *rneΔ645* strains was far more defective at 37ºC than the *rne-1* mutant at 44ºC (Table 3.1, Ow *et al*., 2000). Thus, RNase E is likely to have different affinities for tRNAs, rRNAs, and mRNAs. In addition, based on the wide range of processing rates (Table 3.2), some tRNAs are better substrates than others. While all three mutant proteins retain the catalytic domain, neither the RneΔ610 nor RneΔ645 proteins contain either the arginine-rich-RNA-binding site (ARRBS) or the degradosome scaffolding region (Fig. 3.1) (Vanzo *et al*., 1998; Ow *et al*., 2000). It may be that one of these regions is necessary for efficient binding and catalytic activity on mRNAs but is not as critical for cleavage of either tRNAs or 9S rRNA.

Another point regards the nature of the *rneΔ645* allele. Isolated as a revertant of the extensive RNase E truncation mutant, *rneΔ610*, *rneΔ645* encodes an even shorter polypeptide than its progenitor (Fig. 3.1). Surprisingly, this revertant was able to grow at 44ºC albeit at slower rate than the *rne*$^{+}$ strain. We still do not know whether the isolation of *rneΔ645* was dependent on a *rng::cat rneΔ610* double mutant background or whether it can be isolated in the *rneΔ610* single mutant background. While we do occasionally observe individual colonies that grow within the *rneΔ610* patches on 44ºC replica plates, we cannot rule out that these colonies contain chromosomal suppressor mutations such as were picked up in 7/8 of the original colonies we tested.

More importantly, it was surprising to us that an even shorter version of the RneΔ610 protein would repress the lethality observed at 44ºC. A BLAST search using the amino acids missing in RneΔ645 (418-427; ser leu ser leu ser ile leu arg leu ile) did not

yield any known motifs that would explain why the additional truncation generates a protein that is now able to maintain cell viability. The loss of the additional 35 amino acids could change the conformation leading to a protein that more effectively cleaves tRNAs. Solving the crystal structure of RNase E would help explain this puzzle as well as aiding investigators in defining how the various domains of RNase E interact with RNA substrates.

Taken together, it is clear that RNase E plays a major role in all aspects of RNA processing and decay in *E. coli*, including rRNAs, tRNAs, and mRNAs. It is thus somewhat surprising that only the N-terminal catalytic domain of the protein has been evolutionarily conserved and that some organisms such a *Bacillus subtilis* do not even contain an RNase E homologue (Kaberdin *et al*., 1998).

EXPERIMENTAL PROCEDURES

Bacterial strains

The *E. coli* strains used in this study are listed in Table 3.5. To make strain SK9953 (*rneΔ1018::bla rng::cat thyA715 rph-1*/ pQLK16 [*rne*$^+$ Km^r]), bacteriophage P1 mediated transduction was used with SK9206 (*rneΔ1018::bla thyA715 rph-1*/ pQLK16 [*rne*$^+$ Km^r]) as the recipient strain and GW11 (*cafA*/*rng*::*cat* Cm^r *zce726*::Tn*10* Tc^r) as the donor strain. Tranductants were selected for chloramphenicol resistance. SK9953 was subsequently made *recA*$^-$ by P1-transduction using JC10240 (*recA56 srl-300::*Tn*10* Tc^r) as the donor strain. In this case, transductants were selected for tetracycline resistance and scored for sensitivity to UV radiation in order to create SK9954 (*rneΔ1018::bla rng::cat thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pQLK26 [*rne*$^+$ Km^r]). Using *recA*$^-$ strains in subsequent plasmid displacements eliminated possible complications arising from homologous recombination between the various *rne* alleles.

To make SK10148 (*thyA715 rph-1 Δrnt::kan* Km^r), P1-phage transduction was employed using MG1693 (*thyA715 rph-1*) as the recipient strain and STL4519 (*ara-14 galK2 lacY1 mtl-1 xylA5 kdgK51 argE3 hisG4 leuB6 Δ(gpt-proA)62 thr-1 thi-1 rpoS396 rpsL31 glnV44 tsx-33 recB21 recC22 sbcB15 sbcC201 mgl-51 Δrnt::kan* Km^r *racO qsr'O*

rfbD1) as the donor strain. The *Δrnt::kan* allele is a deletion/substitution mutation. Desired transductants were selected for resistance to kanamycin.

Plasmid construction

Vector pMOK18 was made by inserting a *Sma*I/*Sma*I streptomycin resistance cassette from pKRP13 (Reece and Phillips, 1995) into the *Sca*I site of pWSK29 (Wang and Kushner, 1991). To make pMOK19 (*rneΔ610* Sm^r), a *Kpn*I/*Sac*I fragment from pMOK15 (*rneΔ610* Cm^r; Ow *et al*., 2000) containing the *rneΔ61*0 allele was inserted into the *Kpn*I/*Sac*I sites of pMOK18. pMOK21 (*rneΔ645* Sm^r) was isolated naturally from a 44°C replica plate of SK9982 (*thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pMOK19 [*rneΔ610* Sm^r]). pMOK29 (*rneΔ645* Cm^r) was made using site-directed mutagenesis using pMOK15 as the template.

Plasmid displacement

Plasmid displacements were done as described previously (Ow *et al*., 2000). Selection for displacement was done by growing transformants with shaking in Luria broth containing thymine (50 μg/ml) and the antibiotic encoded by the incoming plasmid (either streptomycin [20 μg/ml] or chloramphenicol [20 μg/ml]) overnight at 37°C. Transformants that were resistant to the drug carried on the incoming plasmid were tested for their sensitivity to the drug encoded by the former resident plasmid. For instance, to make SK9982 (*rneΔ1018::bla rng::cat thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pMOK19 [*rneΔ610* Sm^r]), the resident plasmid pQLK26 (*rne*$^+$ Km^r) in SK9954 was displaced with plasmid pMOK19 (*rneΔ610* Sm^r) in LB with thymine and streptomycin. Similarly, to make SK9987 (*rneΔ1018::bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pMOK21 [*rneΔ645* Sm^r]), plasmid pMOK21 (*rneΔ645* Sm^r) was transformed into SK9714 (*rneΔ1018::bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pSBK1 [*rne*$^+$ Cm^r]) and displacement of pSBK1 was promoted by growing transformants in rich liquid medium containing thymine and streptomycin. Strain SK10103 (*rneΔ1018::bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/

pMOK29 [*rneΔ645* Cm^r]) was made by transforming pMOK29 into SK9705 (*rneΔ1018::bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pQLK26 [*rne*$^+$ Km^r]), growing transformants in the appropriate medium, and selecting for chloramphenicol-resistant, kanamycin-sensitive transformants.

Growth curves

Cultures were routinely grown in Luria broth with thymine (50 μg/ml) and chloramphenicol (20 μg/ml), kanamycin (50 μg/ml), or streptomycin (20 μg/ml) at 37^{o}C with agitation until they reached Klett 40-45 (No. 42 green filter; 1 x 10^8-1.1 x 10^8 cells/ml). On reaching this density, cultures were shifted to 44^{o}C. Every 30 minutes thereafter, cells were diluted with fresh pre-warmed medium. Klett values were adjusted by the appropriate dilution factor.

DNA sequencing

Sequencing of the *rneΔ645* allele was done using an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems) as instructed by the manufacturer using primers RNE+240, RNE+292, RNE+747, RNE+1006, and RNE+3628 (Table 3.5). These primers encompassed the *rneΔ610* and *rneΔ645* genes in their entirety.

Site-directed mutagenesis

The introduction of a stop codon into plasmid pMOK15 (*rneΔ610*) to generate the *rneΔ645* allele (pMOK19) was done with the U.S.E. Mutagenesis Kit (Amersham Pharmacia) following the instructions of the manufacturer. The selection and the mutagenesis (which converts the codon for serine 418 from TCG to a TAG stop codon) primers were RNE-893 and RNE+1593T (Table 3.5), respectively. DNA sequencing at the site of the C to A transversion in addition to RNase E Western blot analysis on strain SK10103, were performed to verify the authenticity of the mutation.

Western analysis

Cultures (25 ml) were grown with shaking at 37°C in Luria broth with thymine (50 μg/ml), chloramphenicol (20 μg/ml) or streptomycin (20 μg/ml) to Klett 80 (2 x 10^8 cells/ml), sedimented, and washed in 12.5 ml of cold 50 mM Tris pH 8.0. Pellets were weighed and resuspended in 800 μl of Lysis Buffer (50 mM Tris pH 8.0, 25% sucrose, 0.6 mg/ml lysozyme) per 1 gram of cells. The samples were then iced for 25 minutes followed by the addition of equal volume of Buffer D (200 mM NaCl, 20 mM Tris pH 7.2, 16 mM $MgCl_2$, 2 mM DTT, 2 mM EDTA, 2% nonidet P40 [Sigma], 1% sodium deoxycholate, 1 mM phenylmethylsulphonyl fluoride [PMSF; Sigma], 4 μg/ml aprotinin [Sigma], 0.8 μg/ml leupeptin, 0.8 μg/ml pepstatin [Roche], 20 units/ml DNase I [Roche]) and centrifugation at 15,500 rpm at 4°C for 30 minutes. Protein concentrations in the supernatants were determined using the Bradford method (Bradford, 1976) using bovine serum albumin as the standard. Supernatants were boiled for 5 min in equal volume of SDS/PAGE loading buffer (60 mM Tris pH 6.8, 2% ß-mercaptoethanol, 2% SDS, 10% glycerol, 0.01% bromophenol blue). Fifty μg of each lysate was electrophoresed in an 8% SDS/PAGE gel followed by electrotransferring to a PVDF membrane (Immobilon P; Millipore). Visualization of the RNase E proteins was accomplished using an RNase E MAP antibody raised against the first 20 amino acids of the N-terminus (Ow *et al*., 2000).

Northern analysis

For RNA isolation and Northern analysis, we followed the procedures described in O'Hara *et al*., (1995). Total RNAs used for the tRNA processing Northern blots were obtained from 44°C cultures diluted as described for the growth curves. RNA was obtained from exponentially growing cultures that had been shifted to 44°C for 2 hrs (SK9937) and 4 hrs (SK9714, SK9957, and SK9987). These times were chosen based on the times after shift that the *rne-1* and *rneΔ610* strains stopped growing (Fig. 3.3). RNA from CA265 (wild-type), CAN20-12E/18-11 (RNase II^-, D^-, BN^-, T^-), MG1693 (*rph-1*) and SK10148 (*Δrnt::kan rph-1*)were harvested from exponentially growing cultures at 37°C.

Band densities for half-life determinations were quantitated with a Phosphorimager series 400 (Molecular Dynamics). Decay rates were calculated using linear regression analysis.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the NIGMS (GM57220) to S. R. K. M. C. O. was supported in part by a NIH predoctoral traineeship from NIGMS (GM07106). We are thankful to M. Wachi, M. Deutscher, and S. Lovett for providing strains GW11, CAN20-12E/18-11, and STL4519, respectively. We also thank B. Mohanty and T. Perwez for critical readings of the manuscript.

REFERENCES

Altman, S., L. Kirsebom, and S. Talbot. 1995. Recent studies of RNase P. In: Söll, D., and U. RajBhandary, eds. *tRNA: Structure, Biosynthesis, and Function*. American Society for Microbiology Press, Washington, D. C. pp. 67-78.

Arraiano, C. M., S. D. Yancey, and S. K. Kushner. 1988. Stabilization of discrete mRNA breakdown products in *ams pnp rnb* multiple mutants of *Escherichia coli* K-12. J. Bacteriol. 170: 4625-4633.

Babitzke, P., and S. R. Kushner. 1991. The *ams* (altered mRNA stability) protein and ribonuclease E are encoded by the same structural gene of *Escherichia coli*. Proc. Natl. Acad. Sci. USA 88: 1-5.

Berlyn, M.K. 1998. Linkage map of *Escherichia coli* K-12, edition 10: the traditional map. Microbiol. Mol. Biol. Rev. 62: 814-984.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

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

Casarégola, S., A. Jacq, D. Laoudj, G. Mcgurk, S. Margarson, M. Tempête, V. Norris, and I. B. Holland. 1992. Cloning and analysis of the entire *Escherichia coli ams* gene. *ams* is identical to *hmp1* and encodes a 114 kDa protein that migrates as a 180 kDa protein. J. Mol. Biol. 228: 30-40.

Casarégola, S., A. Jacq, D. Laoudj, G. Mcgurk, S. Margarson, M. Tempête, V. Norris, and I. B. Holland. 1994. Cloning and analysis of the entire *Escherichia coli ams* gene. *ams* is identical to *hmp1* and encodes a 114 kDa protein that migrates as a 180 kDa protein. J. Mol. Biol. 238: 867.

Csonka, L. N., and A. J. Clark. 1980. Construction of an Hfr strain useful for transferring *recA* mutations between *Escherichia coli* stains. J. Bacteriol. 143: 529-530.

Deutscher M.P., C. W. Marlor, and R. Zaniewski. 1985. RNase T is responsible for the end-turnover of tRNA in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 82: 6427-6430.

Deutscher, M. P. 1995. tRNA processing nucleases. In: Söll, D., and U. RajBhandary, eds. *tRNA: Structure, Biosynthesis, and Function*. American Society for Microbiology Press, Washington D. C. pp. 51-65.

Deutscher, M. P., and Z. Li. 2001. Exoribonucleases and their multiple roles in RNA metabolism. Prog. Nucleic Acid Res. Mol. Biol. 66: 67-105.

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

Hajnsdorf, E., F. Braun, J. Haugel-Nielsen, J. Le Derout, and P. Régnier. 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.

Kaberdin, V. R., A. Miczak, J. S. Jakobsen, S. Lin-Chao, K. J. McDowall, and A. von Gabain. 1998. The endoribonucleolytic N-terminal half of *Escherichia coli* RNase E is evolutionarily conserved in *Synechocystis* sp. and other bacteria but not the C-terminal half, which is sufficient for degradosome assembly. Proc. Natl. Acad. Sci. USA 95: 11637-11642.

Kelly, K. O., and M. P. Deustcher. 1992. The presence of only one of five exoribonucleases is sufficient to support the growth of *Escherichia coli*. J. Bacteriol. 174: 6682-6684.

Li, Z., and M. P. Deutscher. 1996. Maturation pathways for *E. coli* tRNA precursors: a random multienzyme process *in vivo*. Cell 86: 503-512.

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.

López, P. J., I. Marchand, S. A. Joyce, and M. Dreyfus. 1999. The C-terminal half of RNase E, which organizes the *Escherichia coli* degradosome, participates in mRNA degradation but not rRNA processing *in vivo*. Mol. Microbiol. 33: 188-199.

Mackie, G. A. 1991. Specific endonucleolytic cleavage of the mRNA for ribosomal protein S20 of *Escherichia coli* requires the product of the *ams* gene *in vivo* and *in vitro*. J. Bacteriol. 173: 2488-2497.

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

McDowall, K. J., R. G. Hernandez, S. Lin-Chao, and S. N. Cohen. 1993. The *ams-1* and *rne-3071* temperature sensitive mutations in the *ams* gene are in close proximity to each other and cause substitutions within a domain that resembles a product of the *Escherichia coli mre* locus. J. Bacteriol. 175: 4245-4249.

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

Mudd, E. A., H. M. Krisch, and C. F. Higgins. 1990. RNase E, an endoribonuclease, has a general role in the chemical decay of Escherichia coli mRNA: evidence that *rne* and *ams* are the same genetic locus. Mol. Microbiol. 4: 2127-2135.

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

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., H. Causton, E. A. Mudd, and C. F. Higgins. 1994. A protein complex mediating mRNA degradation in *Escherichia coli*. Mol. Microbiol. 14: 727-729.

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.

Ray, B.K., and D. Apirion. 1981a. Transfer RNA precursors are accumulated in *Escherichia coli* in the absence of RNase E. Eur. J. Biochem. 114: 517-524.

Ray, B. K., and D. Apirion. 1981b. RNAase P is dependent on RNAase E action in processing monomeric RNA precursors that accumulate in an RNAase E- mutant of *Escherichia coli*. J. Mol. Biol. 149: 599-617.

Reece, K. S., and G. J. Phillips. 1995. New plasmids carrying antibiotic-resistance cassettes. Gene 165: 141-142.

Régnier, P., and E. Hajnsdorf. 1991. Decay of mRNA encoding ribosomal protein S15 of *Escherichia coli* is initiated by an RNase E-dependent endonucleolytic cleavage that removes the 3' stabilizing stem and loop structure. J. Mol. Biol. 217: 283-292.

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

Taraseviciene, L., A. Miczak, and D. Apirion. 1991. The gene specifying RNase E (*rne*) and a gene affecting mRNA stability (*ams*) are the same gene. Mol. Microbiol. 5: 851-855.

Wachi, M., G. Umitsuki, and K. Nagai. 1997. Functional relationship between *Escherichia coli* RNase E and the CafA protein. Mol. Gen. Genet. 253: 515-519.

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

Wang, R.F., and S. R. Kushner. 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and expression in *Escherichia coli*. Gene 100: 195-199.

Table 3.1. mRNA half-lives at 37°C.

Transcript	Half-life (min)[a]			
	rne$^+$	*rne-1*[b]	*rneΔ610*	*rneΔ645*
rpsO[c]	1.8 ± 0.4	2.9 ± 0.3	16.3 ± 1.7	9.4 ± 2.1
rpsT[c]	1.5 ± 0.1	2.3 ± 0.4	4.4 ± 0.3	2.9 ± 0.0

[a] The data represent the average of two independent determinations at 37°C in rich medium.

[b] *rne-1* encodes a temperature-sensitive RNase E protein that is unable to support cell viability at 44°C (Ono and Kuwano, 1979, Babitzke and Kushner, 1991).

[c] For *rpsO*, the half-life of the *rpsO2* transcript (Ow *et al*., 2000) is presented. For *rpsT*, the half-life is the average of the two major transcripts (Mackie, 1991).

Table 3.2. Processing of polycistronic and monocistronic tRNA transcripts.

Transcript		Half-life[a] (min)			
	Region	*rne*$^+$	*rne-1*	*rneΔ610*	*rneΔ645*
glyW cysT leuZ	cysT-leuZ	ND	9.9 ± 0.5	7.0 ± 0.4	3.5 ± 0.7
	glyW-cysT[b]	ND	5.9	NC[c]	NC[c]
argX hisR leuT proM	hisR-leuT	ND	14.5 ± 1.9	15.0 ± 5.4	4.1 ± 0.1
	argX-hisR[d]	ND	12.5	10.4	3.4
tyrT tyrV tpr	tyrT 5' spacer[e]	ND	7.7	3.1	4.1
	tyrT-tyrV[f]	ND	8.9	9.4	8.1

pheU	pheU tRNA	ND	31.8 ± 5.0	20.2 ± 3.0	8.6 ± 3.1

[a] Half-lives were determined as the average of two independent experiments for the largest precursor detected in each Northern blot.

ND: no intermediates were detected.

[b, d, e, f] Experiments were done once.

[c] NC: half-lives were not calculated because of the low level of intermediates that were observed.

Table 3.3. Relative amounts of mature tRNAs in various *rne* mutants.

Gene	$tRNA^{Cys}$	$tRNA^{His}$	$tRNA_1^{Tyr}$	$tRNA^{Lys}$	$tRNA^{Phe}$
rne$^+$	1.00	1.00	1.00	1.00	1.00
rneΔ645	0.93	0.97	0.86	1.00	0.67
rneΔ610	0.70	0.59	0.72	0.61	0.49
rne-1	0.66	0.59	0.35	0.41	0.46

The membranes that were probed for mature tRNAs were quantitated with a Phosphoimager series 400 (Molecular Dynamics). For each tRNA, the average of the value obtained from the *rne*$^+$ strain was set at 1.00 and divided into the values obtained for the three *rne* mutants. These values represent the total cellular levels of each tRNA with the exception of $tRNA^{Lys}$.

Table 3.4. *E. coli* strains and plasmids.

Strain	Genotype	Source or reference
CA265	*lacZ125 tyrT66 trpA49 relA1*	*E. coli* Genetic Stock Center
CAN20-12E/18-11	*lacZ125 tyrT66 trpA49 relA1 rna⁻ rnd⁻ rnb⁻ rbn⁻ rnt⁻*	Deutscher *et al*. (1985)
GW11	*cafA/rng*::*cat* Cm^r *zce726*::Tn*10* Tc^r	Wachi *et al*. (1997)
JC10240	*recA56 srl300::*Tn*10* Tc^r	Csonka and Clark (1980)
MG1693	*thyA715 rph-1*	*E. coli* Genetic Stock Center
SK9206	*rneΔ1018::bla thyA715 rph-1* / pQLK26 (*rne*$^+$ Km^r)	Ow *et al*. (2000)
SK9705	*rneΔ1018::bla thyA715 rph-1 recA56 srl300::*Tn*10* Tc^r/	
	pQLK26 (*rne*$^+$ Km^r)	Ow *et al*. (2000)
SK9714	*rneΔ1018::bla thyA715 rph-1 recA56 srl300::*Tn*10* Tc^r/	
	pSBK1 (*rne*$^+$ Cm^r)	Ow *et al*. (2000)
SK9937	*rneΔ1018::bla thyA715 rph-1 recA56 srl300::*Tn*10* Tc^r/	
	pMOK13 (*rne-1* Cm^r)	Ow *et al*. (2000)
SK9953	*rneΔ1018::bla rng::cat thyA715 rph-1* / pQLK26 (*rne*$^+$ Km^r)	This study
SK9954	*rneΔ1018::bla rng::cat thyA715 rph-1 recA56 srl300::*Tn*10* Tc^r/	

	pQLK26 (*rne*$^{+}$ Km^{r})	This study
SK9957	*rneΔ1018::bla thyA715 rph-1 recA56 srl300::*Tn*10* Tc^{r}/	
	pMOK15 (*rneΔ610* Cm^{r})	Ow *et al*. (2000)
SK9982	*rneΔ1018::bla rng::cat thyA715 rph-1 recA56 srl300::*Tn*10* Tc^{r}/	
	pMOK19 (*rneΔ610* Sm^{r})	This study
SK9987	*rneΔ1018::bla thyA715 rph-1 recA56 srl300::*Tn*10* Tc^{r}/	
	pMOK21 (*rneΔ645* Sm^{r})	This study
SK10103	*rneΔ1018::bla thyA715 rph-1 recA56 srl300::*Tn*10* Tc^{r}/	
	pMOK29 (*rneΔ645* Cm^{r})	This study
SK10148	*thyA715 rph-1 Δrnt::kan* Km^{r}	This study
STL4519	*ara-14 galK2 lacY1 mtl-1 xylA5 kdgK51 argE3 hisG4 leuB6*	
	Δ(gpt-proA)62 thr-1 thi-1 rpoS396 rpsL31 glnV44 tsx-33	
	recB21 recC22 sbcB15 sbcC201 mgl-51 Δrnt::kan Km^{r} *racO*	
	qsr'O rfbD1	Susan Lovett

Plasmids	Genotype	Source or reference
pKRP13	High-copy plasmid with Sm^r	Reece and Phillips (1995)
pWSK29	6-8 copy plasmid with Ap^r	Wang and Kushner (1991)
pMOK15	6-8 copy plasmid with *rneΔ610* Cm^r	Ow *et al*. (2000)
pMOK18	6-8 copy plasmid with Sm^r; made by cloning the Sm^r cassette (*Sma*I/*Sma*I) from pKRP13 into the *Sca*I site of the Ap^r gene in pWSK29	This study
pMOK19	6-8 copy plasmid with *rneΔ610* Sm^r; made by cloning a *Kpn*I/*Sac*I fragment containing *rneΔ610* from pMOK15 into the *Kpn*I/*Sac*I sites in pMOK18	This study
pMOK21	6-8 copy plasmid with *rneΔ645* Sm^r	This study
pMOK29	6-8 copy plasmid with *rneΔ645* Cm^r	This study

Table 3.5. Oligonucleotides used in this study.

Primer	Sequence
argX-hisR	5' tactaccaccgcagctcaag 3'
cysT	5' acggatttgcaatccgctac 3'
cysT-leuZ	5' gctcgggaagaaagtg 3'
glyW-cysT	5' ttcagccagacatccgcttg 3'
hisR	5' cccacgacaactggaatcac 3'
hisR-leuT	5' attgtcacaacttctaataa 3'
PB5SRNA	5' cgctacggcgtttcacttct 3'
pheU2	5' ggactcggaatcgaaccaag 3'
RNE-893	5' aagaacgctaggcctttgccaccct 3'
RNE+1593T	5' gaatagagagcgacagctattcgttgtcacgcacggt 3'
RNE+240	5' cctcacggttatcgtcagctc 3'
RNE+292	5' ctagacacgaggccatcgg 3'
RNE+747	5' gtagctatctggttctg 3'
RNE+1006	5' gagcaacgtaatcgttc 3'
RNE+3628	5' cattgttggttagcaaggatgccattc 3'
tyrSP	5' cgctcgaatcgaaccttagt 3'
tyrT5'A	5' acggggtaatgctttttactggcctgctcccttatcgggaagcg 3'
tyrTV-3'	5' cgaaccttcgaagtcgatga 3'

Figure 3.1. Schematic representation of the *rneΔ610* and *rneΔ645* alleles. The *rneΔ610* mutation encodes an internally deleted RNase E protein encompassing the first 427 amino acids of the N-terminus plus the last 25 amino acids of the C-terminus (Ow *et al*., 2000). The *rneΔ645* revertant contains a C to A transversion on serine 418 (T**C**G → T**A**G) generating a premature stop codon. The resultant RNase E protein, RneΔ645, is 35 amino acids shorter than the RneΔ610 protein. The RneΔ645 polypeptide contains only the first 417 amino acids of the N-terminus. The figure is not drawn to scale.

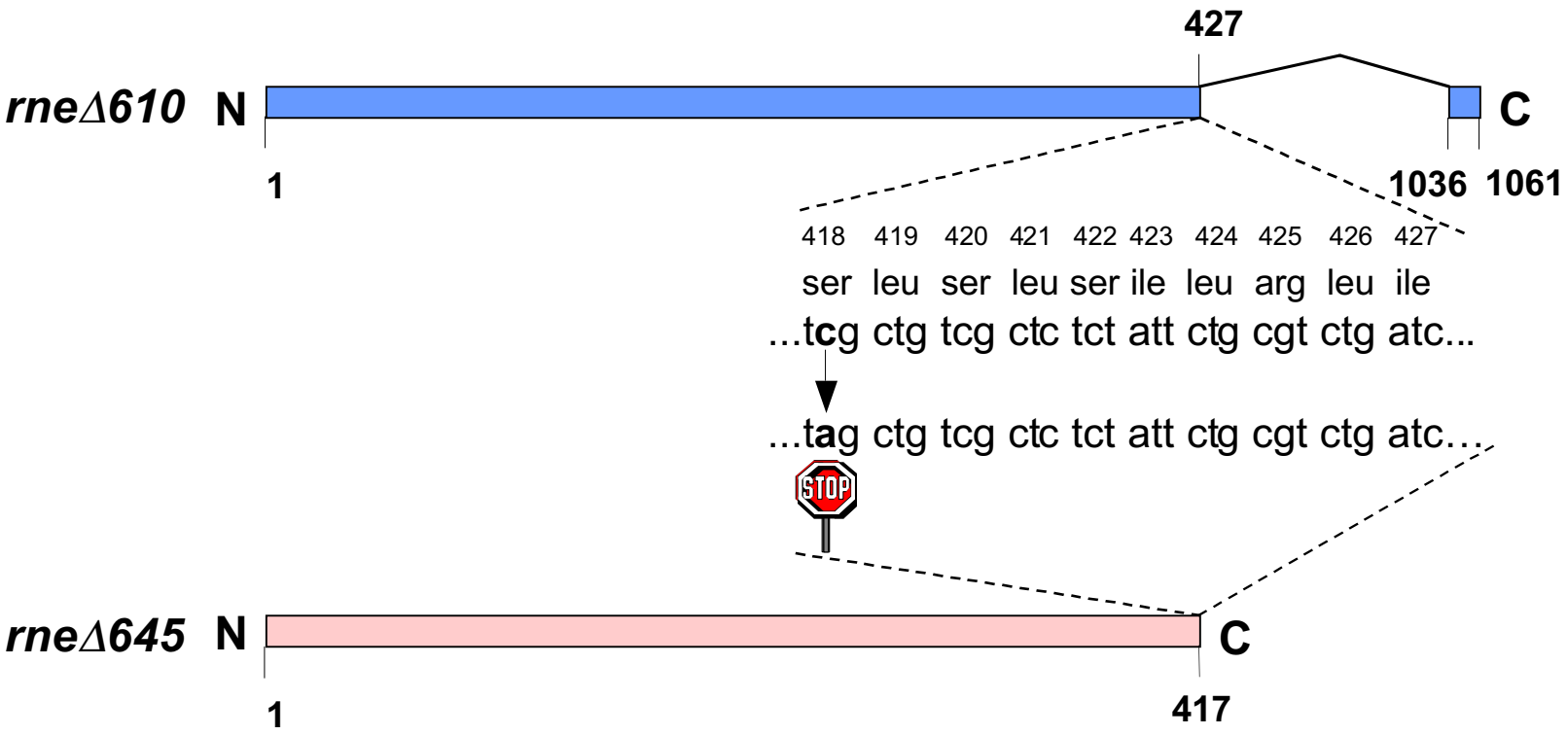

427
rneΔ610
N
C
1
1036
1061
418 419 420 421 422 423 424 425 426 427
ser leu ser leu ser ile leu arg leu ile
...tcg ctg tcg ctc tct att ctg cgt ctg atc...
...tag ctg tcg ctc tct att ctg cgt ctg atc...
STOP
rneΔ645
N
C
1
417

Figure 3.2. Western analysis of various RNase E proteins. Equivalent amounts of total protein lysates (50 μg) were electrophoresed in an 8% SDS/PAGE and electrotransferred onto an Immobilon PVDF membrane (Millipore). Membranes were probed with an RNase E MAP antibody raised against the first 20 amino acids of the N-terminus (Experimental Procedures). Size standards are on the left. Asterisks denote the RNase E proteins. Lane 1, SK9714; lane 2, SK9937; lane 3, SK9957; lane 4, SK9987.

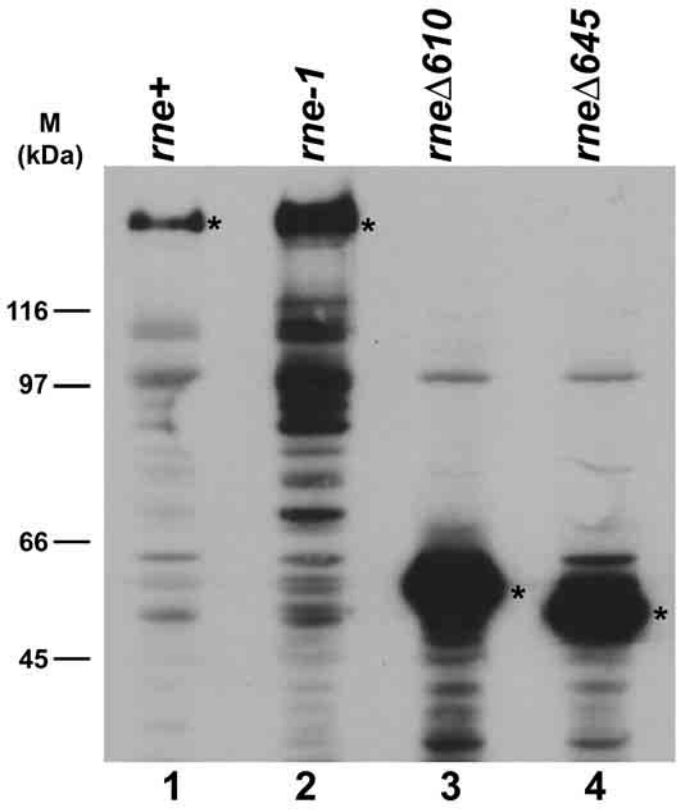

M
(kDa)
rne+
rne-1
rneΔ610
rneΔ645
116
97
66
45
*
*
*
*
1
2
3
4

Figure 3.3. Comparison of the growth properties of strains carrying the *rne*$^{+}$, *rne-1*, *rneΔ610*, and *rneΔ645* alleles. Overnight standing cultures were diluted into fresh L broth + thymine (50 μg/ml) and grown with shaking at 37ºC until reaching Klett 40-45 (1 x 10^{8}-1.1 x 10^{8} cells/ml). The cultures were then shifted to a shaking 44ºC water bath. Every 30 min thereafter, cultures were diluted by one half with fresh pre-warmed medium. Klett readings taken during each 30 min interval were adjusted with the appropriate dilution factor. SK9937 (x ; *rne-1*) ; SK9957 (o; *rneΔ610*) ;SK9714 (❑; *rne*$^{+}$) and SK9987 (Δ; *rneΔ645*).

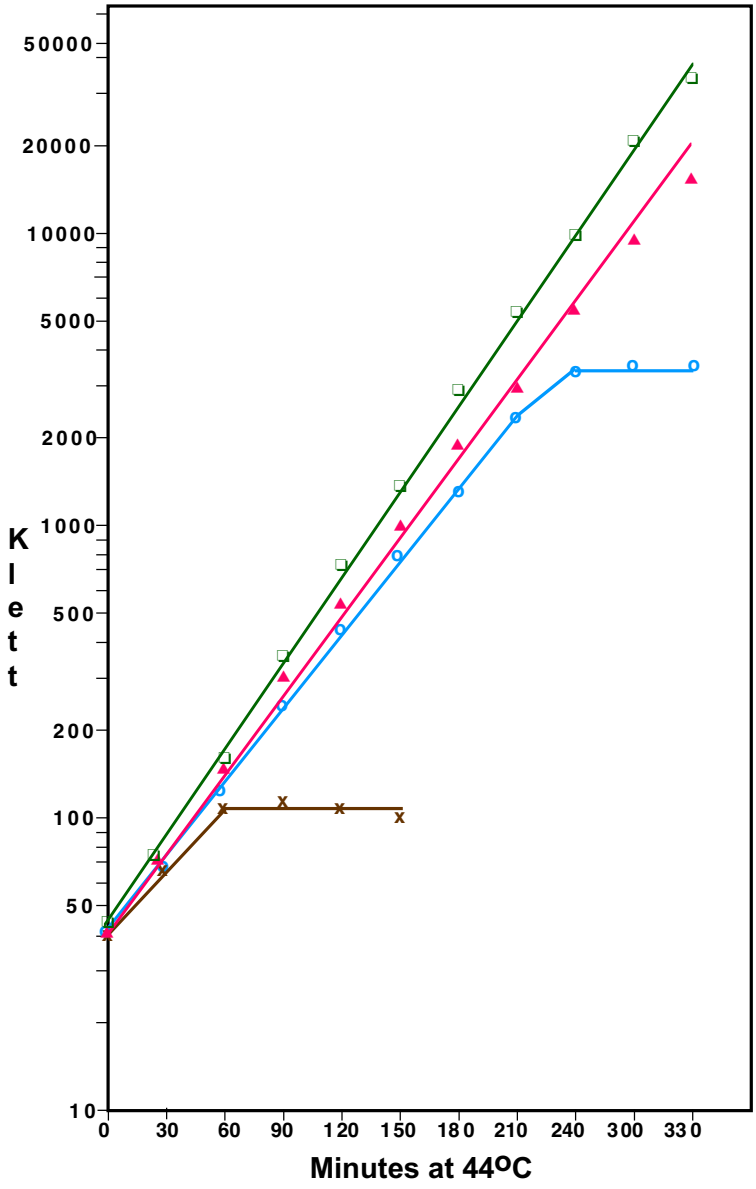

50000
20000
10000
5000
2000
1000
500
200
100
50
10
K
l
e
t
t
0
30
60
90
120
150
180
210
240
300
330
Minutes at 44°C

Figure 3.4. Northern analysis of the 5S rRNA. Five μg of total steady-state RNA isolated from 37°C cultures growing in LB medium (lanes 1-4) were separated by 6% PAGE/7 M urea and electrotransferred onto a nylon membrane (MSI MagnaCharge; Osmonics) and probed with a ^{32}P 5' end-labeled PB5SRNA primer (Table 3.5) complementary to the 5S rRNA. Steady-state RNA at 44°C was obtained by shifting exponentially cultures growing at 37°C to the high temperature for 70 minutes (lanes 5-8). The arrow indicates the 5S rRNA while the larger species are processing intermediates. Lanes 1, 5: SK9714; lanes 2, 6: SK9937; lanes 3, 7: SK9957; lanes 4, 8: SK9987.

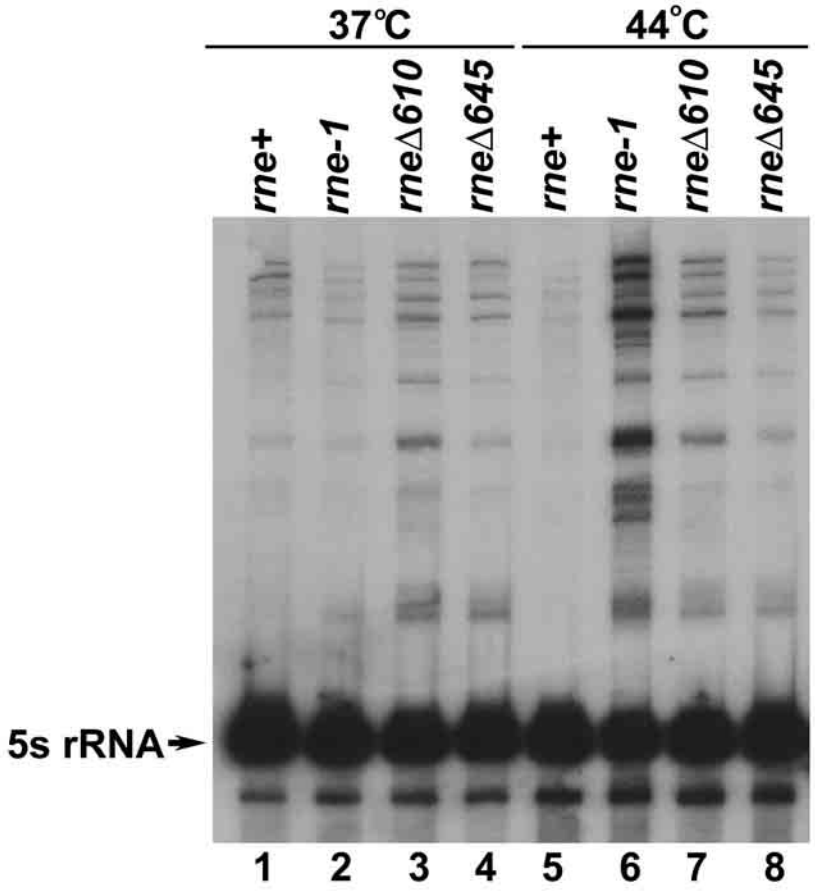

37℃
44℃
rne+
rne-1
rneΔ610
rneΔ645
rne+
rne-1
rneΔ610
rneΔ645
5s rRNA
1
2
3
4
5
6
7
8

Figure 3.5. Processing of the *glyW cysT leuZ* polycistronic transcript. Cultures were grown in LB medium at 44°C as described in Experimental Procedures. Ten μg of total RNA isolated from SK9714 (*rne*$^+$), SK9937 (*rne-1*), SK9957 (*rneΔ610*), and SK9987 (*rneΔ645*) at 0, 2, 5, 10, 20, and 30 minutes after the addition of rifampicin was loaded in each lane. The RNA samples were electrophoresed by a 6% PAGE/7 M urea gel and electrotransferred onto a nylon membrane (MSI MagnaCharge; Osmonics). The same membrane was used for the three Northern blots shown in this figure by following the recommendations of the membrane's manufacturer for stripping. (A) Schematic of the *glyW cysT leuZ* transcript. Numbers indicate the size (nt) of the spacer regions 5' and 3' of the $tRNA^{Cys}$ gene. Hatched rectangles indicate the oligonucleotide probes. All oligonucleotide probes were 5' end-labeled with ^{32}P. The diagram is not drawn to scale. (B) Processing of the 3' end of $tRNA^{Cys}$. Northern analysis was done using an oligonucleotide probe (cysT-leuZ; Table 3.5) complementary to the 12 nt long spacer region between $tRNA^{Cys}$ and $tRNA^{Leu}$. (C) Processing of the 5' end of $tRNA^{Cys}$. The Northern blot was stripped and reprobed with oligonucleotide glyW-cysT (Table 3.5) complementary to a section of the 54 nt long 5' spacer region of $tRNA^{Cys}$. (D) Detection of the mature $tRNA^{Cys}$ using the cysT probe (Table 3.5). Sizes of selected bands are indicated on the right.

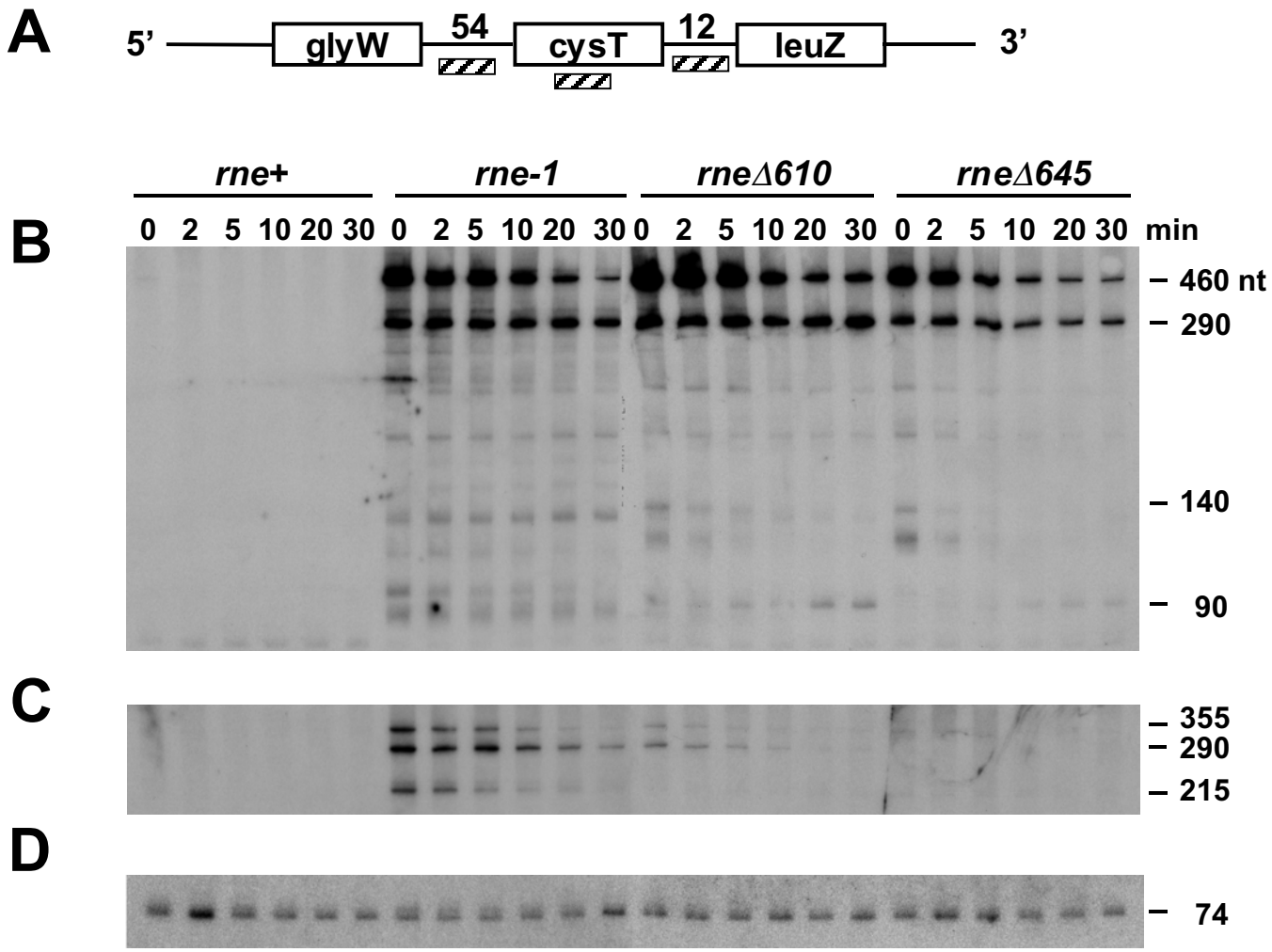

A
5'
glyW
54
cysT
12
leuZ
3'
B
rne+
rne-1
rneΔ610
rneΔ645
0 2 5 10 20 30 0 2 5 10 20 30 0 2 5 10 20 30 0 2 5 10 20 30 min
460 nt
290
140
90
C
355
290
215
D
74

Figure 3.6. Processing of the *argX hisR leuT proM* polycistronic transcript. The same procedures were followed as described in Figure 3.5. (A) Schematic of the *argX hisR leuT proM* polycistronic transcript. The lengths of the spacers between the tRNA genes are numbered in the diagram. Hatched rectangles indicate the oligonucleotide probes. The diagram is not drawn to scale. (B) Processing of the 3' end of $tRNA^{His}$. Oligonucleotide hisR-leuT (complementary to the 20 nt spacer region between $tRNA^{His}$ and $tRNA^{Leu}$) (Table 3.5) was used to probe for the unprocessed 3' spacer of $tRNA^{His}$. (C) Processing of the 5' end of $tRNA^{His}$. The blot was stripped and reprobed with primer argX-hisR (Table 3.5). (D) Northern analysis of the mature $tRNA^{His}$. Sizes of selected bands are indicated on the right.

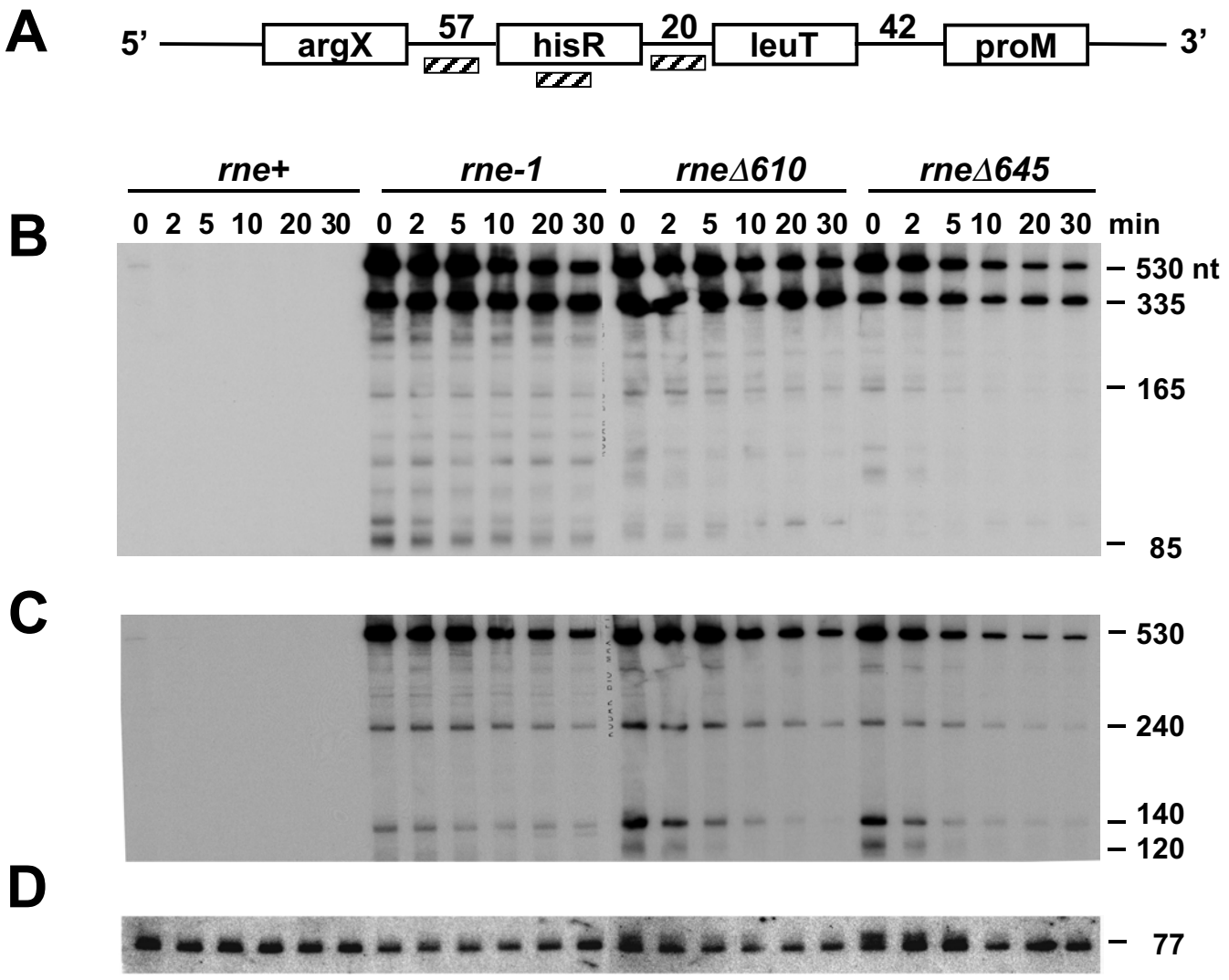

A
5'
argX
57
hisR
20
leuT
42
proM
3'
B
rne+
rne-1
rneΔ610
rneΔ645
0 2 5 10 20 30 0 2 5 10 20 30 0 2 5 10 20 30 0 2 5 10 20 30 min
530 nt
335
165
85
C
530
240
140
120
D
77

Figure 3.7. Processing of the *tyrT tyrV tpr* polycistronic transcript. The same procedures were followed as described in Figure 3.5 except that the time points were 0, 2, 10, and 20 minutes. Hatched rectangles indicate the oligonucleotide probes. (A) Schematic of the *tyrT tyrV tpr* polycistronic transcript. The lengths of the spacers between the tRNA genes are numbered in the diagram. Hatched rectangles indicate the oligonucleotide probes. (B) Processing of the 3' end of $tRNA_1^{Tyr}$. Northern analysis was conducted using oligonucleotide tyrSP (Table 3.5) complementary to a section of the 209 long spacer region between $tRNA_1^{Tyr}$ and $tRNA_1^{Tyr}$. (C) Processing of the 5' end of $tRNA_1^{Tyr}$. Northern analysis was done using primer tyrT5'A (Table 3.5) specific to the 44 nt long 5' unprocessed termini of $tRNA_1^{Tyr}$. (D) Detection of the mature $tRNA_1^{Tyr}$ using primer tyrTV3' (Table 3.5). The sizes of various tRNA transcripts are indicated on the right.

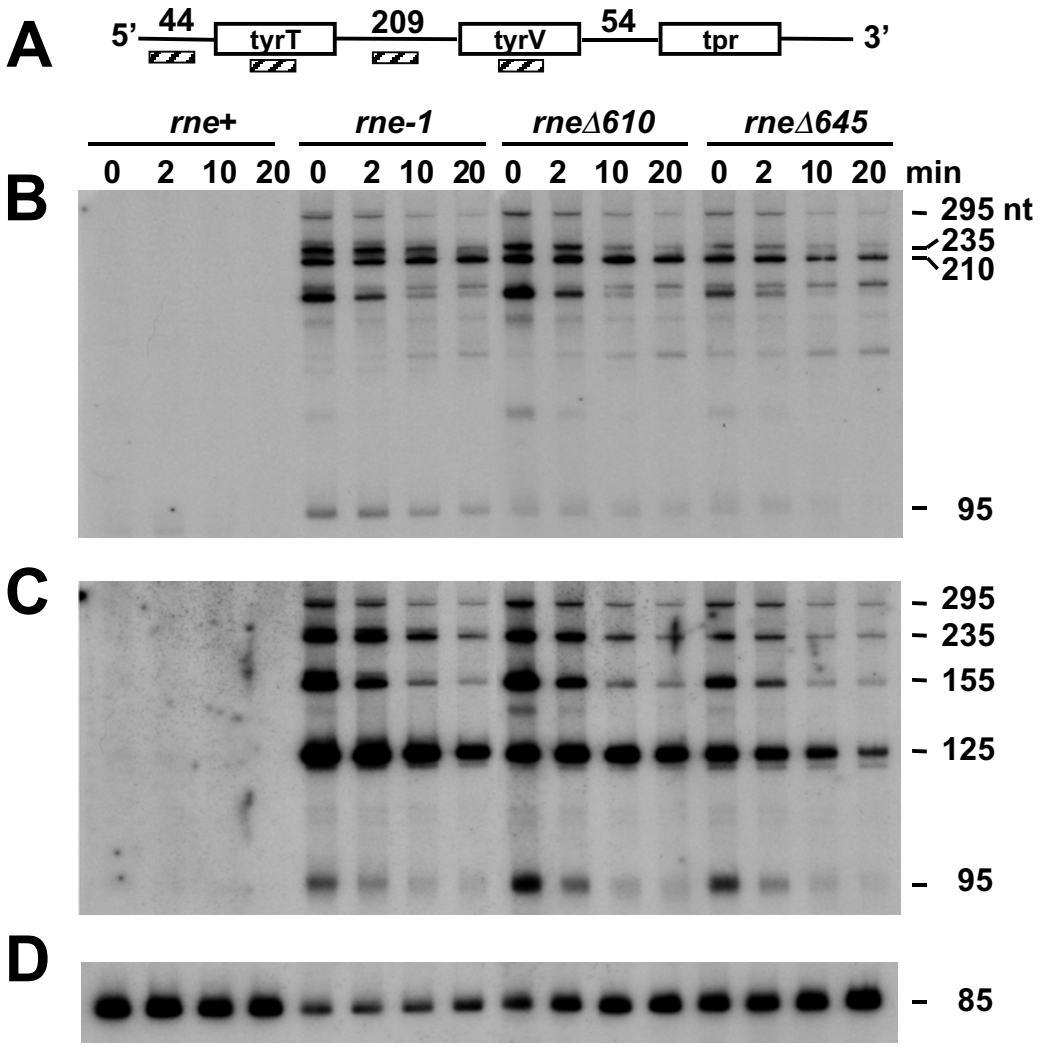

A
5'
44
tyrT
209
tyrV
54
tpr
3'
B
rne+
rne-1
rneΔ610
rneΔ645
0 2 10 20 0 2 10 20 0 2 10 20 0 2 10 20 min
295 nt
235
210
95
C
295
235
155
125
95
D
85

Figure 3.8. Processing of the *pheU* monocistronic transcript. The same conditions were used as described in Figure 3.5. (A) Schematic of the *pheU* monocistronic transcript. The hatched rectangle is the oligonucleotide probe (pheU2; Table 3.5) complementary to the mature $tRNA^{Phe}$. Diagram is not drawn to scale. (B) Northern analysis using the pheU2 oligonucleotide probe (Table 3.5) to detect both the mature $tRNA^{Phe}$ as well as the unprocessed $tRNA^{Phe}$ precursor. The sizes of both species are indicated on the right.

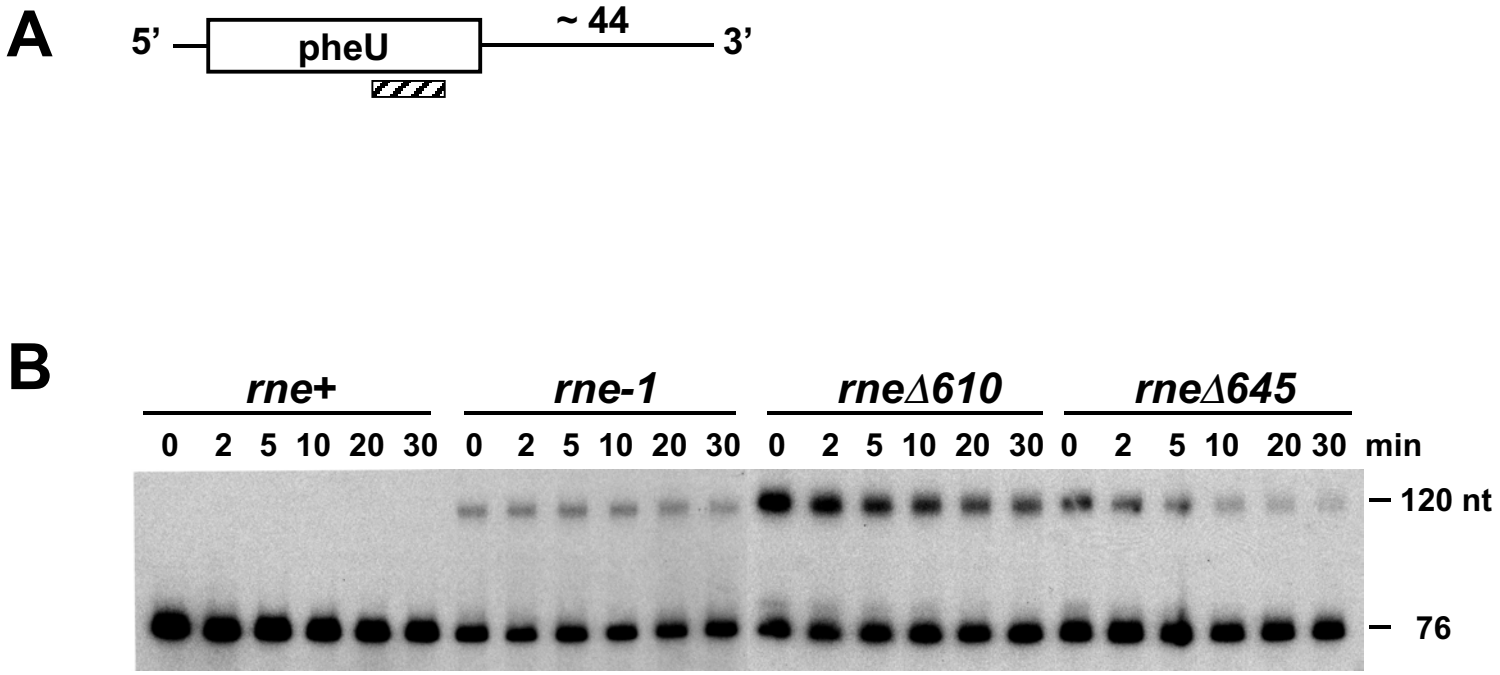
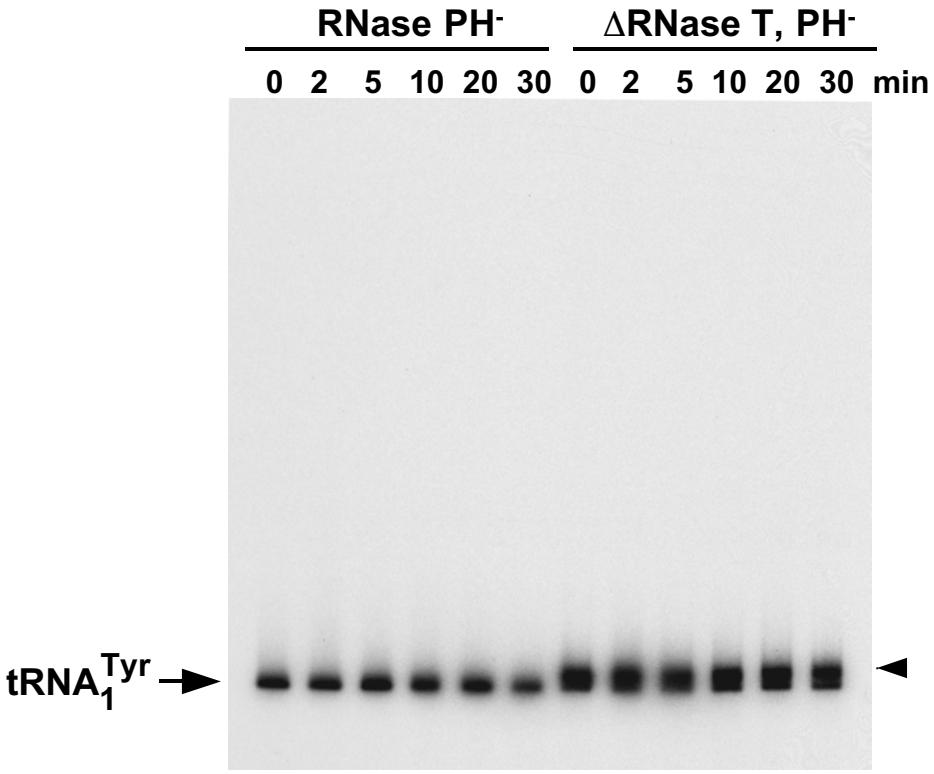

A
5’
pheU
~ 44
3’
B
rne+
rne-1
rneΔ610
rneΔ645
0 2 5 10 20 30 0 2 5 10 20 30 0 2 5 10 20 30 0 2 5 10 20 30 min
120 nt
76

Figure 3.9. Processing of $tRNA_1^{Tyr}$ in an RNase T RNase PH double mutant. Cultures of MG1693 (*rph-1*) and SK10148 (*Δrnt::kan rph-1*) were grown exponentially at 37ºC and samples were harvested at 0, 2, 5, 10, 20, and 30 minutes after adding rifampicin. Total RNA (10 μg) extracted from each sample was electrophoresed by a 6% PAGE/7M urea gel and electrotransferred onto a nylon membrane (MSI MagnaCharge; Osmonics). The Northern blot was probed with tyrTV3' (complementary to the mature $tRNA_1^{Tyr}$; Fig. 3.7a; Table 3.5). Arrowhead on right denotes the slightly longer precursor that occurs in an RNase T^- RNase PH^- strain (Li and Deutscher, 1996).


RNase PH-
ΔRNase T, PH-
0 2 5 10 20 30 0 2 5 10 20 30 min
tRNA1Tyr

CHAPTER 4

TRANSCRIPTION OF THE *ESCHERICHIA COLI rne* GENE FROM THREE DISTINCT PROMOTERS IS REQUIRED FOR AUTOREGULATION, NORMAL mRNA DECAY, AND CELL GROWTH[1]

[1]Ow, M. C., Q. Liu, M. E. Andrew, V. F. Maples, and S. R. Kushner. Submitted to Molecular Microbiology, 7/11/01.

ABSTRACT

The *rne* gene of *Escherichia coli* encodes RNase E, an essential endoribonuclease that is involved in both mRNA decay and rRNA processing. Here we present evidence that the gene is transcribed from three promoters, p1, p2, and p3. The p2 and p3 promoters map 34 and 145 nt upstream from the previously characterized *rne* promoter, p1, generating unusually long 5' UTRs of 395 and 506 nt, respectively. Based on promoter/*lacZ* transcriptional fusions, p1 is stronger than either p2 or p3. Low copy number or single copy number vectors carrying *rne* transcribed from either p1, p2, or p3 alone complement the *rneΔ1018::bla* deletion mutation at 30°C, 37°C, and 44°C. However, normal autoregulation requires the presence of all three promoters. There is a direct correlation between promoter strength, intracellular levels of RNase E, the half-lives of the *rpsO* and *rpsT* mRNAs and growth rates. Specifically, reduction in transcription leads to reduced RNase E levels, longer mRNA half-lives, and slower growth rates.

INTRODUCTION

The *rne* gene of *Escherichia coli* encodes RNase E, an essential endoribonuclease that plays a major role in the degradation of messenger RNAs and the processing of ribosomal RNA (Coburn and Mackie, 1999; Régnier and Arraiano, 2000; Steege, 2000). Biochemical experiments have shown that RNase E exists as a component of a multi-protein complex, called the degradosome, that includes the exoribonuclease polynucleotide phosphorylase (PNPase), the RhlB RNA helicase, and the glycolytic enzyme enolase (Carpousis *et al*., 1994; Py *et al*., 1994; 1996; Miczak *et al*., 1996; Blum *et al*., 1997). Although recent evidence suggests that the degradosome forms *in vivo* (Liou *et al*., 2001), analysis of various *rne* deletion alleles has shown that degradosome assembly is not necessary for either rRNA processing or mRNA decay at 37°C (López *et al*., 1999; Ow *et al*., 2000). Given that RNase E plays an integral role in several aspects of RNA metabolism and that the presence of the *rne* gene on high copy number plasmids impairs cell growth (Claverie-Martin *et al*., 1991), it is reasonable to expect that the cellular concentration of this

enzyme will be tightly regulated. Indeed, RNase E appears to be maintained at a constant level by a mechanism of autoregulation that involves the degradation of its own transcript. For example, the half-life of the *rne* mRNA varies from one min in a wild-type strain to greater that 10 min in a strain carrying the temperature-sensitive *rne-1* allele (Henry *et al*., 1992; Jain and Belasco, 1995; Diwa *et al*., 2000). Thus, in wild-type strains, increased *rne* gene dosage does not result in a proportionate accumulation of RNase E protein due to the decreased stability of the *rne* transcript. In contrast, in a *rne-1* mutant the mRNA half-life increases along with a concomitant increase in protein level (Mudd and Higgins, 1993).

Primer extension analysis of the *rne* transcript has shown that it contains an unusually long (361 nt) 5' untranslated region (UTR) (Claverie-Martin *et al*., 1991). Subsequently, Jain and Belasco (1995) demonstrated that autoregulation was in part mediated in *cis* by the presence of the 5' UTR. Based on chemical alkylation experiments, the 5' UTR was shown to fold into a series of secondary structures (Diwa *et al*., 2000). Furthermore, despite considerable DNA sequence divergence, the secondary structure of the noncoding 5' leader region has been highly conserved amongst the *rne* mRNAs from different gram-negative bacteria (Diwa *et al*., 2000). Interestingly, although the 5' UTR contains at least one RNase E cleavage site, autoregulation does not appear to be dependent on cleavage at this site (Jain and Belasco, 1995; Liu, Ow, and Kushner, manuscript in preparation). Recently, the C-terminal region of RNase E has also been implicated in the autoregulation of the protein (Jiang *et al*., 2000; Ow *et al*., 2000).

In light of the tight control of *rne* expression, we became interested in the role of the 5' UTR. When we compared primer extension products obtained from a *rne-1* mutant with a wild-type strain, we detected two extra 5' termini that mapped upstream of the previously characterized p1 promoter. In this communication, we report the identification and characterization of two additional *rne* promoters, p2 and p3. The transcriptional start sites of p2 and p3 lie 34 and 145 nt upstream of the p1 start site, resulting in 5' UTRs of 395 and 506 nt, respectively. The functionality of p2 and p3 was confirmed by transcription reporter assays and promoter deletion mutations. Any one of the promoter constructions in 6-8

copies or in single copy provided enough transcriptional activity to support cell viability in a *rneΔ1018::bla* deletion strain. However, the level of RNase E protein was reduced between 3-100-fold in strains where the expression of the *rne* gene was driven by only one of the three promoters. The decay of the *rpsO* and *rpsT* transcripts, two mRNAs that require RNase E for degradation, was slowed at both 37°C and 30°C. In terms of changes in growth rate and mRNA decay, the most striking effects were seen at 30°C. In addition, deletion of any two of the three promoters led to either a partial or complete loss of autoregulation.

RESULTS

Identification of the 5' termini of the *rne* transcript

Previous experiments in wild-type *E. coli* have demonstrated a transcription start-site (p1) at a G residue 361 nt upstream from the AUG start codon of the *rne* transcript (Claverie-Martin *et al*., 1991; Jain and Belasco, 1995) (Fig. 4.1) as well as two RNase E cleavage sites located 49 and 50 nt downstream of the p1 transcriptional-start site (Fig. 4.1). A similar pattern of termini was observed at either 30°C or 42°C (Fig. 4.1). When an identical primer extension was carried out on total RNA isolated from the temperature-sensitive *rne-1* mutant, there was a significant increase in the amount of the p1 transcript at both 30°C and 42°C compared to the wild-type control (Fig. 4.1). The observed cleavage at the RNase E sites was also expected at 42°C because the Rne-1 protein still retains some residual activity at this temperature. Surprisingly, however, two additional signals upstream of p1 were consistently observed (Fig. 4.1, lanes 3, 4). These bands were located 34 and 145 nt upstream of p1, respectively, and were tentatively named p2 and p3. The p2 and p3 products could also be seen in the wild-type strain but only after very long exposure times (data not shown).

Inspection of the nucleotide sequence in the region upstream of p1 revealed two potential promoters (Fig. 4.2) that could account for the primer extension products observed in Fig. 4.1. For the putative p2 promoter, the -35 (GTGAGT) and -10 (TAAAAG)

sequences were 49% similar to the consensus σ^{70} promoter (Mulligan *et al*., 1984). With p3, the -35 (TTCGCG) and -10 hexamers (TATATT) were 39% identical. Interestingly, the -35 box of p1 overlapped with the transcriptional-start-site of the putative p2 promoter (Fig. 4.2).

Determination of the transcriptional activity associated with p1, p2, and p3 using *lacZ* fusions

Even though we observed primer extension products that were RNA concentration-dependent (Fig. 4.1) and could identify potential σ^{70} promoters by visual inspection of the DNA sequence (Fig. 4.2), we wanted to rule out the possibility that these 5' termini might have arisen from technical artifacts associated with the experiment. Accordingly, we used PCR amplification to generate DNA fragments containing p1, p2, and p3 individually, and in combination, and cloned each fragment upstream of a *lacZ* reporter gene in a multiple copy transcriptional fusion vector, pRS551 (Simons *et al*., 1987). In this way, we generated plasmids pMOK5 (p1-*lacZ*), pMOK9 (p2-*lacZ*), pMOK10 (p3-*lacZ*), and pMOK11 (p1p2p3-*lacZ*) (Table 4.1) that carried *rne* promoter-*lacZ* transcriptional fusions. As a negative control, we amplified a DNA fragment (-169 to -262) (Fig. 4.2) that only contained the -35 box of p3 and also inserted it into pRS551 (pMOK12, p0-*lacZ*). When these plasmids were transformed into MC1061 (*ΔlacX74*), a high level of β-galactosidase activity was detected in all of the transformants except for those carrying pMOK12 (data not shown).

In order to differentiate the level of β-galactosidase activity produced by each of the promoters, we made single copy isolates of each construct by using the transducing lambda phage system developed by Simons *et al*. (1987). The fusions present in plasmids pMOK5, pMOK9, pMOK10, pMOK11, and pMOK12 were transferred to either λRS45 or λRS74 by a double recombination event (Simons *et al*., 1987) to generate λMOK1, λMOK2, λMOK3, λMOK4, and λMOK5 (Table 4.1). NK7049 (*ΔlacX74*) was then

lysogenized with the recombinant phages to construct strains carrying a single copy of each promoter-*lacZ* fusion.

As shown in Fig. 4.3, there was not a direct correlation between β-galactosidase activity and the predicted promoter efficiency based on homology to the σ^{70} promoter consensus sequence. The p1 promoter (58%) had the highest level of activity (Fig. 4.3) followed by p3 (39% homology) and p2 (49% homology). The lysogen containing all three promoters displayed the highest level of β-galactosidase activity, although the increase was less than the sum of each of the individual promoters (Fig. 4.3). No significant amount of β-galactosidase activity was observed in the p0-*lacZ* control.

Construction of *rne* promoter deletion mutations

While the experiments described above indicated that all three promoters were transcriptionally active, we wanted to determine if they functioned *in vivo* to direct the synthesis of RNase E. Since constructing promoter deletion mutations in the chromosome is technically difficult, we decided to use a complementation approach. First, a series of promoter deletions was generated (Fig. 4.4) and initially cloned into low copy number vectors (6-8 copies) such that the full-length *rne* gene was transcribed by either p1, p2, or p3 (see Experimental Procedures). To verify that transcription was arising from a single promoter in the pMOK25 (*rneΔp2Δp3* Km^r), pMOK28 (*rneΔp1Δp3* Km^r), and pMOK30 (*rneΔp1Δp2* Km^r) constructs (Fig. 4.4, Table 4.1), we performed primer extension analysis in a strain carrying a 294 nt chromosomal deletion in the 5' UTR of *rne* (*rneΔ208*). By using primers complementary to the region that had been deleted from the 5' leader region in *rneΔ208* (Fig. 4.4), the observed primer extension products generated were specific to the plasmid encoded *rne* constructs. The results of this analysis confirmed that the *rneΔp2Δp3* and *rneΔp1Δp2* plasmids each generated a single 5' terminus that coincided with either the p1 or p3 start-sites, respectively, identified in Fig. 4.1 (data not shown). In the case of the *rneΔp1Δp3* mutant, two new 5' termini, located at +4 and +6 (Fig. 4.2) were detected. These arose because in this construction the -35 and -10 sequences of the p2 promoter were

left intact but the normal transcription start site at -34 had been deleted since it was part of the -35 sequence of p1 (Figs. 4.2, 4.4). With pSBK6 (*rneΔp1Δp2Δp3* Cm^r) no primer extension products were observed.

We then took advantage of the *rneΔ1018::bla* deletion mutant (Ow *et al*., 2000) to determine if the various *rne* genes could support cell viability. Either SK9714 (*rneΔ1018::bla recA56*/ pSBK1 [rne^+ Cm^r]) or SK9705 (*rneΔ1018::bla recA56*/ pQLK26 [rne^+ Km^r]) was transformed with the plasmids carrying the various promoter constructs. Because of plasmid incompatibility, we were able to select for the incoming 6-8 copy number plasmid and displace the resident plasmid if the incoming *rne* gene was transcribed at a level sufficient to support cell viability. Plasmids pMOK25 (*rneΔp2Δp3* Km^r), pMOK28 (*rneΔp1Δp3* Km^r), and pMOK30 (*rneΔp1Δp2* Km^r) were able to displace the wild-type gene, while pSBK6 (*rneΔp1Δp2Δp3* Cm^r) was not.

Once we established the validity of the promoter deletion mutations, we also cloned each allele into a mini-F plasmid vector. In this fashion, single copy plasmids pMOK44 (rne^+ Sp^r), pMOK41 (*rneΔp1Δp2* Sp^r), pMOK42 (*rneΔp1Δp3* Sp^r), pMOK43 (*rneΔp2Δp3* Sp^r), or pMOK40 (a single copy Sp^r vector) were constructed as described in Experimental Procedures. The single copy constructions were introduced into the *rne* deletion strain employing a two step procedure. Initially, the mini-F plasmids (pMOK41-45, Table 4.1) were transformed into SK9714 (*rneΔ1018::bla*/ pSBK1 [rne^+ Cm^r]). Transformants that became Sp^r were subsequently transformed with the 6-8 copy Km^r vector pWSK129 (Wang and Kushner, 1991) so as to displace the resident plasmid pSBK1 (rne^+ Cm^r) carrying the same replicon. In all cases, except for the pMOK40 control, the single copy plasmids were able to complement the *rneΔ1018::bla* deletion strain.

RNase E levels in single copy promoter deletion strains

Because RNase E has been shown to autoregulate its own synthesis (Jain and Belasco, 1995), we wanted to determine how the decreased transcriptional activity associated with the individual promoters (Fig. 4.3) affected RNase E levels. Specifically, if

autoregulation functioned in the traditional way, reduced transcription of the *rne* gene should initially lead to reduced RNase E protein levels and a concomitant stabilization of its own transcript. This in turn would lead to increased translation of the RNase E protein, helping to maintain the appropriate *in vivo* level. However, Western blot analysis of total protein isolated from SK10143 (*rneΔ1018:bla*/ pMOK44 [rne^{+}]), SK10142 (*rneΔ1018:bla*/ pMOK43 [*rneΔp2Δp3*]), SK10145 (*rneΔ1018:bla*/ pMOK42 [*rneΔp2Δp3*]), and SK10146 (*rneΔ1018:bla*/ pMOK41 [*rneΔp1Δp2*]), demonstrated that RNase E proteins levels decreased between 17-100-fold when the individual promoters were driving *rne* transcription in the single copy vectors (Fig. 4.5, lanes 2, 4, 6, 8). As a normalization control, the membrane was stripped and reprobed for the UvrD protein, an enzyme with no role in RNA metabolism (Fig. 4.5, lanes 2, 4, 6, 8).

Autoregulation of RNase E is impaired in the promoter deletion mutants

The very large drop in RNase E levels associated with transcription from a single promoter (Fig. 4.5) did not fit the existing model of RNase E autoregulation. We, therefore, also tested the same promoter mutants when they were present in 6-8 copies in the *rneΔ1018::bla* genetic background. With the wild-type *rne* gene, autoregulation appeared to function as previously described (Jain and Belasco, 1995). Specifically, even though the copy number increased from one to 6-8, the level of RNase E protein was essentially unchanged (1.04 compared to 1, Fig. 4.5). In contrast, with each of the individual promoter constructions, the level of RNase E increased between 3-10-fold (Fig. 4.5), indicating the loss of autoregulation. However, even though autoregulation was no longer functioning, there was still between a 3.5-34-fold reduction in RNase E protein level (Fig. 4.5, lanes 1, 3, 5, 7) with the individual promoters.

mRNA decay in the promoter deletion mutants

Since transcription from either p1, p2, or p3 alone led to large decreases in RNase E protein levels (Fig. 4.5), we hypothesized that there should be a significant impact on the

decay rates of RNase E-dependent mRNAs. Accordingly, we measured the half-lives of the *rpsO* and *rpsT* mRNAs, two transcripts that have been shown to require RNase E for their degradation (Mackie, 1991; Hajnsdorf *et al*., 1994). Initially, we determined the half-lives of these two transcripts in strains carrying the promoter mutants in 6-8 copies. While there were small increases in both half-lives when only p2 or p3 were present (Table 4.2), a strain with only p1 appeared as efficient in the decay of these two transcripts as the wild-type control (Table 4.2).

Since there was less than a 2-fold change in the *rpsO* and *rpsT* half-lives (Table 4.2) under conditions (*rneΔp1Δp3*) where there was a 33-fold decrease in RNase E protein (Fig. 4.5, lane 5), it appeared that the cell contained a considerable excess of mRNA degrading capacity. To test this hypothesis directly, we measured the half-lives of the *rpsO* and *rpsT* transcripts in strains in which the promoter mutants were in single copy. Under these conditions, RNase E protein levels were reduced up to 100-fold (Fig. 4.5, lanes 4, 6, 8). At 37°C, the half-lives of the *rpsO* and the *rpsT* mRNAs in the single copy wild-type strain, SK10143, increased approximately 1.4-fold compared to its 6-8 copy number counterpart SK9714 at 37°C (Fig. 4.6, Tables 4.2, 4.3a). Reduction of the gene dosage of the various promoter deletions from 6-8 copies to single copy, however, resulted in a much more pronounced effect on the decay of these two mRNAs. This was especially evident in SK10145 (*rneΔp1Δp3*), the strain in which *rne* is being transcribed from only the p2 promoter. At 37°C, the half-life of *rpsO* in SK10145 increased more than 2-fold over the wild-type control SK10143 (2.3 ± 0.3 vs. 5.1 ± 0.5) (Table 4.3a). Smaller increases were observed for SK10146 (*rneΔp1Δp2*) and SK10142 (*rneΔp2Δp3*) (Table 4.3a).

At 30°C, message decay in the wild-type control was slower than at 37°C (Table 4.3b). However, the differences in the half-lives in the promoter deletion mutants compared to the wild-type control were much more dramatic than those at 37°C. For example, in SK10145 (*rneΔp1Δp3*) (Table 4.3b) the *rpsO* half-life was longer than 30 min, a greater than 5-fold increase over the rne^+ control (Table 4.3b, Fig. 4.6). The *rpsT* mRNA in SK10145 decayed with a half-life of 12.4 ± 2.4 min as compared to 4.0 ± 0.7 in the wild-

type strain, a greater than 3-fold increase (Table 4.3b). Smaller increases (1.6-2.2-fold) were observed in SK10146 (*rneΔp1Δp2*) and SK10142 (*rneΔp2Δp3*).

Growth properties of promoter deletion strains

Previous work has shown that RNase E is essential for cell viability (Apirion and Lassar, 1978; Ono and Kuwano, 1979). Although all of the promoter deletion mutants were viable, we were interested in determining whether the reduced RNase E protein levels and concomitant increase in mRNA half-lives affected cell growth. To our surprise, when the various promoter alleles were present in 6-8 copies/cell, all the strains had comparable growth rates (~30 min) at 37ºC in rich medium (data not shown).

We therefore decided to test the strains with the single-copy constructs where RNase E levels in several of the mutants were less than 5% of wild-type levels (Fig. 4.5). We first carried out growth curves at 44ºC, a temperature at which we expected that there would be a greater demand for RNase E activity. Surprisingly, the three promoter deletion strains grew comparably to the wild-type control (Fig. 4.7a). As expected, the temperature-sensitive *rne-1* strain showed a dramatically reduced growth rate under these conditions (Fig. 4.7a). Even at 37ºC, where there were significant differences in the half-lives of the *rpsO* and *rpsT* transcripts (Table 4.3a), the doubling times were all within the 30-34 min range (Fig. 4.7b).

However, in the course of our study, we noticed that strain SK10145 (*rneΔp1Δp3*) grew slower than its 6-8 copy counterpart (SK10102) on solid rich medium at 30ºC. To follow up on this observation, we examined the growth properties of SK10143 (rne^+), SK10146 (*rneΔp1Δp2*), SK10145 (*rneΔp1Δp3*), and SK10142 (*rneΔp2Δp3*) in rich medium at 30ºC. At this temperature SK10143 (rne^+, 58 min) had the shortest generation time followed by SK10142 (*rneΔp2Δp3*, 61 min), SK10146 (*rneΔp1Δp2*, 68 min), and SK10145 (*rneΔp1Δp3*, 86 min) (Fig. 4.7c). Even though the longer generation times suggested a cold-sensitive phenotype, all of the strains containing the single copy promoter transcripts grew at 16ºC on solid rich medium (data not shown).

DISCUSSION

In the experiments described above, we have identified a new feature of the intracellular regulation of RNase E protein levels. Specifically, the gene is transcribed from three independent promoters. The p2 promoter is located 34 nucleotides upstream from the currently characterized promoter, p1 (Fig. 4.2). The transcription-start-site of p2 overlaps with the -35 box of p1. The p3 promoter is 145 nucleotides upstream from the p1 start-site. Transcription stemming from the p2 and p3 promoters generates 5' UTRs of 395 and 506 nucleotides, respectively. Thus, all three transcripts have very long untranslated regions (Fig. 4.2). Interestingly, all three promoters are active individually *in vivo* but each drives expression at different levels, with p1 accounting for most of the *rne* message in the cell followed by p3 and p2 (Fig. 4.3). Although p2 has a higher degree of homology to the consensus σ^{70} promoter (Mulligan *et al*., 1984), the overlap of its transcription start-site with the -35 region of p1, may explain why it works less efficiently than p3 (Fig. 4.3).

While many *E. coli* genes have multiple promoters, what is significant in this case is how they are involved in the regulation of intracellular RNase E levels. Using *rne-lacZ* fusions, Jain and Belasco (1995) have shown that RNase E levels are subject to feedback regulation if the wild-type *rne* gene copy number is increased. In fact, our data with the wild-type *rne* gene supports this conclusion, since a 6-8-fold increase in copy number led to an insignificant increase in RNase E protein level (Fig. 4.5, lanes 1, 2).

The converse of this type of regulation is that reduced RNase E activity should lead to an increase in the half-life of the *rne* transcript thereby increasing the expression of the protein. This has been demonstrated but only in strains in which RNase E activity was reduced by employing a mutationally altered protein encoded by the *rne-1* allele (Jain and Belasco, 1995; Diwa *et al*., 2000). In our experiments, we have reduced the level of transcription of the wild-type *rne* gene by generating constructs in which only one of the three promoters is present. If autoregulation is simply a function of variations in the functional half-live of the *rne* transcript, we would have expected to see comparable levels of RNase E protein in SK10143 (rne^{+}) and SK10142 (*rneΔp2Δp3*) since the transcriptional

activity of the p1 promoter alone was only moderately reduced compared to the wild-type control (Fig. 4.3). It was thus rather surprising that under these conditions there was actually a 10-fold decrease in the level of RNase E protein (Fig. 4.5, lanes 2, 8). The results were even more dramatic with the p2 and p3 promoters (Fig. 4.5, lanes 4, 6). These data suggest that when the level of wild-type RNase E is reduced below a certain threshold, even if the *rne* transcript is stabilized, it does not lead to increased protein levels.

Further support for the proposition that *rne* feedback regulation may not be entirely a function of *rne* transcript stability is provided by the data examining protein levels as a function of plasmid copy number using the single promoter constructs. For example, a 6-8-fold increase in the copy number of the p1 construct led to a 10-fold increase in RNase E protein level (Fig. 4.5, lanes 7, 8). In fact, there were significant increases in the RNase E protein level with each of the single promoter constructs (Fig. 4.5). Thus, autoregulation appears to be lost when the *rne* gene is transcribed from a single promoter even though the 361 nt 5' UTR that is thought to be required for autoregulation (Jain and Belasco, 1995) has been left intact.

While it is not clear how to interpret the discrepancies between the results reported here and the previous work of Jain and Belasco (1995), it should be noted that all their constructs contained both p1 and p2, based on their use of a 1.0 kb *Bcl*I-*Hin*dIII fragment derived from pFMK32 (Claverie-Martin *et al*., 1989). However, it is not obvious why the presence of multiple transcription start-sites would be necessary for normal autoregulation. It would appear, however, that the mechanism of autoregulation is considerably more complicated than previously envisioned. These results also suggest that because of the highly conserved nature of the 5' UTRs in other bacterial species (Diwa *et al*., 2000), there may be additional *rne* promoters in these organisms as well.

A further somewhat unexpected result regards the relationship between RNase E levels and mRNA decay rates. We chose to measure the half-lives of the *rpsO* and *rpsT* transcripts because both have been shown to be degraded in an RNase E-dependent fashion (Mackie, 1991; Hajnsdorf *et al*., 1994). While the half-lives of these two transcripts

increased as a function of reduced RNase E levels, at 37°C there was only a 2.2-fold increase in the *rneΔp1Δp3* construct even though there was less than 5% of the wild-type level of RNase E protein (Table 4.3a, Fig. 4.5, lane 6). In fact, we only saw a large increase in the stability of the two transcripts at 30°C (Table 4.3b) even though the relative levels of the RNase E protein in the four constructs was identical to what we observed at 37°C (Fig. 4.5, lanes 2, 4, 6, 8; data not shown). These data suggest that the presence of the three promoters ensures sufficient RNase E levels to achieve optimum mRNA decay rates. They also indicate that *E. coli* contains a considerable excess of mRNA degrading capacity.

The half-life data also raise an interesting question regarding the loss of autoregulation of RNase E protein levels. The biggest effect on mRNA half-lives was seen in the strain carrying *rneΔp1Δp3* (Table 4.3). In addition, it has been shown previously that the *rne* transcript is degraded by RNase E (Henry *et al.,* 1992; Jain and Belasco, 1995). Although we did not measure the half-life of the *rne* transcript because of technical difficulties, it would be expected that its half-life would have increased in this strain. However, the loss of autoregulation was least pronounced in this genetic background (Fig. 4.5). Furthermore, when the single-promoter constructions were present in 6-8 copies/cell, the *rpsO* and *rpsT* half-lives were close to wild-type levels, suggesting that a change in the half-life of the *rne* transcript could not account for the 3-10-fold increase in RNase E protein levels.

Although there was a correlation between intracellular RNase E levels and mRNA decay rates, a comparable relationship with growth rates was not equally apparent. For example, at 37°C in the presence of 6-8 copies of the *rne* gene, all of the promoter constructs gave generation times that were equivalent within experimental error (data not shown), even though there was a 3.5-33-fold reduction in RNase E protein levels (Fig. 4.5). When the constructs were present in single-copy, there were larger reductions in RNase E levels and significant differences in the *rpsO* and *rpsT* half-lives at 37°C (Table 4.3a), but the generation times of the three mutants and the wild-type control were still almost identical (Fig. 4.7b). There also were no large differences in the growth rates of these strains at 44°C

(Fig. 4.7a). It was only at 30°C that phenotypic differences among the promoter deletion strains and the wild-type control became more evident (Fig. 4.7c).

These observations were exactly opposite to what we had expected. Specifically, since growth rates are a direct function of temperature, we had hypothesized a greater need for RNase E at 44°C compared to either 37°C or 30°C. Therefore, with less than 5% of wild-type RNase E protein in the strains containing only p2 or p3, we anticipated significant growth defects at the elevated temperature, not at 30°C as was actually observed (Fig. 4.7). Perhaps even more surprising was the fact that even under these conditions, the cells grew remarkably well. While an explanation for this behavior is not apparent at the present time, the results provide further support for the hypothesis of Ow *et al*. (2000) that defects in mRNA decay are not why RNase E is required for cell viability.

Clearly, the transcriptional regulation of *rne* is more sophisticated than originally imagined and provides further evidence for how the cellular concentration of this crucial endoribonuclease is modulated. While it would appear that feedback regulation is employed to keep RNase E levels from becoming potentially toxic, this type of control may only function to insure a certain optimum concentration. Since there appears to be excess mRNA degrading capacity, reduced level of RNase E may not be as serious an issue.

EXPERIMENTAL PROCEDURES

Bacterial strains

The *E. coli* K-12 strains used in this work are listed in Table 4.1.

Plasmid constructions

Primers used for the various PCR constructions are described in Table 4.4. Plasmids containing the various *rne* promoters were PCR-amplified from pQLK26 (*rne*$^+$ Km^r) (Ow *et al*., 2000). Primers morneP1a and morneP1b were used to amplify a 165 base pair (bp) fragment containing the p1 promoter (nucleotides -66 to +99; numbered as in Fig. 4.2); morneP2a and morneP2b-1 were used to PCR amplify a 128 bp fragment with the p2

promoter (nucleotides -143 to -16); morneP3a-1 and morneP3b-1 were used to amplify a 125 bp fragment containing the p3 promoter (nucleotides -204 to -80); morneP3a-1 and morneP1b were used to PCR amplify a 301 bp fragment containing all the promoters (nucleotides -204 to +99). Primers morneP3a and prneX were used to amplify a 94 bp region, referred to as p0, upstream (nucleotides -262 to -169) but including the -35 hexamer of the p3 promoter, which was used as our negative control. Each set of primers was designed to contain *EcoR*I and *Bam*HI sites for subsequent insertion upstream of the *lacZ* reporter gene in the transcriptional fusion vector pRS551 (Km^r; Simons *et al*., 1987) to create pMOK5 (p1-*lacZ* Km^r), pMOK9 (p2-*lacZ* Km^r), pMOK10 (p3-*lacZ* Km^r), pMOK11 (p1p2p3-*lacZ* Km^r), pMOK12 (p0-*lacZ* Km^r). The sequence of each PCR insert was confirmed by DNA sequencing.

pQLK26 (*rne*$^+$ Km^r) and pSBK1 (*rne*$^+$ Cm^r) are 6-8 copy plasmids encoding the wild-type *rne* gene (Ow *et al*., 2000). pMOK22 (*rneΔp2* [a deletion of p2 from nucleotides -122 to -42 as numbered in Fig. 4.2] Km^r) was made by the overlap extension PCR method described by Ho *et al*. (1989) using Platinum *Pfx* DNA polymerase (Life Technologies) with primers RNE-1014, RNEP2ARMB, RNEP2ARMC, and RNE+3628. pMOK27 (*rneΔp3* [deletion of nucleotides -193 to -146] Km^r) was made using primers RNE-1014, RNEP3ARMB, RNEP3ARMC, and RNE+3628. pMOK30 (*rneΔp1Δp2* [deletion of nucleotides -78 to -2] Km^r) was made with primers RNE-1014, P1P2ARMB, P1P2ARMC, and RNE+3628. The promoter deletions in pMOK22, pMOK27, and pMOK30 were generated using the wild-type *rne* gene in pQLK26 as the template. pMOK28 (*rneΔp1Δp3* [deletion of nucleotides -193 to -146 and -39 to -2] Km^r) was made with primers RNE-1014, RNEP1ARMB, RNEP1ARMC, and RNE+3628 using pMOK27 as the template. pMOK 25 (*rneΔp2Δp3* [deletion of nucleotides -193 to -146 and -78 to -42] Km^r) was made with primers RNE-1014, RNEP3ARMB, RNEP3ARMC, and RNE+3628 using pMOK22 as the template. pSBK6 (*rneΔp1Δp2Δp3* [deletion of nucleotides -193 to -146 and -78 to -2] Cm^r) was made from pMOK30 using primers RNE-1014, RNEP3ARMB, RNEP3ARMC, and RNE+3628. Primers RNE-1014 and RNE+3628 were engineered to

contain *Eco*RI and *Xba*I sites. All PCR fragments were cloned into the *Eco*RI and *Xba*I sites in pWSK129 (Km^r), a 6-8 copy plasmid (Wang and Kushner, 1991), except for pSBK6 which was cloned into pSBK29 (Cm^r), also a 6-8 copy plasmid (Mohanty and Kushner, 1999). All promoter lesions were confirmed by sequencing the junction of each deletion.

The single copy vector, pVMK94, was constructed by digesting pWSK29 (Wang and Kushner, 1991) with *Bgl*II. A 2.3 kb DNA fragment containing the *bla* (Ap^r) gene as well as the multiple cloning sites of the *lacZα* gene was gel-purified and treated with the Klenow fragment of DNA polymerase I. Vector pBK22 (Babitzke and Kushner, 1991) was digested with *Asp*718, and a 3.3 kb DNA fragment containing the mini-F single copy origin of DNA replication was gel-purified and treated with the Klenow fragment of DNA polymerase I. The 2.3 kb and the 3.3 kb DNA fragments from pWSK29 and pBK22, respectively, were ligated to create pVMK94. Plasmids pMOK35 (*rneΔp1Δp2*), pMOK36 (*rneΔp1Δp3*), and pMOK37 (*rneΔp2Δp3*) were constructed by inserting the *Eco*RI/*Not*I fragments harboring the various alleles of *rne* from pMOK30, pMOK28, and pMOK25, respectively, into the *Eco*RI/*Not*I sites of pVMK94. Inserting the *Sac*I/*Kpn*I fragment from pSBK1 containing the rne^+ allele into the *Sac*I/*Kpn*I sites of vector pVMK94 resulted in pMOK38 (rne^+). pMOK39 (*rne-1*) was made by ligating a *Sac*I/*Kpn*I DNA fragment from pMOK13 into the *Sac*I/*Kpn*I sites of pVMK94.

In order to change the *bla* drug marker in the single-copy plasmids for subsequent transformation into the *rneΔ1018::bla* deletion background, we inserted a *Hin*dII/*Hin*dII spectinomycin (Sp^r) resistance cassette from pKRP13 (Reece and Phillips, 1995) into the unique *Sca*I site of the *bla* gene in pVMK94, pMOK35, pMOK36, pMOK37, pMOK38, and pMOK39 to generate pMOK40, pMOK41, pMOK42, pMOK43, pMOK44, and pMOK45, respectively.

Lysogen construction

To make single copy *rne* promoter-*lacZ* fusions, we transferred each construct into the chromosome by way of transducing lambda phages λRS45 or λRS74. The fusions in strains SK9738 (F$^-$ *araD139 Δ(ara-leu)7697 ΔlacX74 galE15 galK16 hsdR2 rpsL strA*/ pMOK5 [p1-*lacZ* Km^r]), SK9742 (F$^-$ *araD139 Δ(ara-leu)7697 ΔlacX74 galE15 galK16 hsdR2 rpsL strA*/ pMOK9 [p2-*lacZ* Km^r]), SK9743 (F$^-$ *araD139 Δ(ara-leu)7697 ΔlacX74 galE15 galK16 hsdR2 rpsL strA*/ pMOK10 [p3-*lacZ* Km^r]), and SK9744 (F$^-$ *araD139 Δ(ara-leu)7697 ΔlacX74 galE15 galK16 hsdR2 rpsL strA*/ pMOK11 [p1p2p3-*lacZ* Km^r]) were transferred to λRS45, while the fusion in SK9745 (F$^-$ *araD139 Δ(ara-leu)7697 ΔlacX74 galE15 galK16 hsdR2 rpsL strA*/ pMOK12 [p0-*lacZ* Km^r]) was transferred to λRS74 via a double recombination event as described by Simons *et al*. (1987). Plaques containing the recombinant lambda phages, λMOK1 (p1-*lacZ*), λMOK2 (p2-*lacZ*), λMOK3 (p3-*lacZ*), and λMOK4 (p1p2p3-*lacZ*) were isolated based on their blue plaque phenotype while λMOK5 (p0-*lacZ*) was identified by its white plaque phenotype following standard lambda procedures described by Silhavy *et al*. (1984).

These recombinant lambda phages were used to lysogenize NK7049 (*ΔlacX74 galOP308 rpsL*) to form SK9726 (*ΔlacX74 galOP308 rpsL* λMOK1 [p1-*lacZ*]), SK9701 (*ΔlacX74 galOP308 rpsL* λMOK2 [p2-*lacZ*]), SK9702 (*ΔlacX74 galOP308 rpsL* λMOK3 [p3-*lacZ*]), SK9703 (*ΔlacX74 galOP308 rpsL* λMOK4 [p1p2p3-*lacZ*]), and SK9704 (*ΔlacX74 galOP308 rpsL* λMOK5 [p0-*lacZ*]). SK9726, SK9701, SK9702, and SK9703 were selected as blue colonies on LB plates containing kanamycin (50 μg/ml) and X-gal (5-bromo-4-chloro-indolyl-ß-D-galactoside) at 40 mM. SK9704 was selected as a white colony on the same medium. The integrity of each of the fusion in the lysogens was confirmed by chromosomal DNA sequencing.

Plasmid displacement

Plasmid displacements were done essentially as described previously using SK9714 or SK9705 (Ow *et al*., 2000) as the recipient strains. Displacements to create strains with the 6-8 copy plasmids encoding an *rne* allele were carried out for 6-12 hrs with shaking at 37°C in Luria broth containing thymine (50 μg/ml) and chloramphenicol (20 μg/ml) or kanamycin (50 μg/ml) to select for the incoming plasmid. Displacements to generate strains carrying the various *rne* alleles in single copy were done by first transforming SK9714 with pMOK44 (*rne*$^+$ Sp^r), pMOK41 (*rneΔp1Δp2* Sp^r), pMOK42 (*rneΔp1Δp3* Sp^r), pMOK43 (*rneΔp2Δp3* Sp^r), or pMOK39 (*rne-1* Sp^r). Transformants that became spectinomycin-resistant (20 μg/ml) were then subsequently transformed with pWSK129 (Km^r 6-8 copy vector). Selection was done at 37°C in rich medium containing kanamycin for 12-72 hours to promote the loss of resident plasmid pSBK1 (*rne*$^+$ Cm^r). Thus, strains SK10143 (*rne*$^+$), SK10146 (*rneΔp1Δp2*), SK10145 (*rneΔp1Δp3*), SK10142 (*rneΔp2Δp3*), and SK10144 (*rne-1*) each carried a single-copy plasmid that encoded a particular *rne* allele as well as the vector pWSK129. The presence of each *rne* promoter construct was confirmed by DNA sequencing.

ß-Galactosidase assays

Cultures were grown in 20 ml of Luria broth containing kanamycin (50 μg/ml) until they reached a density of 1.25 x 10^8 cells/ml. The cultures were pelleted at 4°C, resuspended in 5 ml of ice-cold Z Buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM M KCl, 1 mM $MgSO_4$, pH 7.0) (Miller, 1972), and centrifuged again. The pellets were then redissolved in 250 μl of Z Buffer supplemented with 10 mM PMSF and 0.2 M DTT. The cell suspensions were sonicated twice with 20 second bursts followed by centrifugation at 4°C for 10 minutes at 13,000 rpm. The protein concentration of each supernatant fraction was determined using the Bradford method (1976) with BSA as the standard.

ß-galactosidase assays were similar to those described by Miller (1972). Zero to 125 μg of protein extract obtained by sonication were added to 1 ml of Z Buffer followed

by 200 μl of ONPG (o-nitrophenyl-ß-D-galactoside, 20 mg/ml in 60 mM Na_2HPO_4 and 40 mM NaH_2PO_4, pH 7.0). ONPG hydrolysis was carried out for one hour at room temperature and was stopped with 500 μl of 1 M $NaCO_3$. The level of o-nitrophenol, a byproduct of ONPG hydrolyzation, was measured at OD_{420}. ß-galactosidase activity was determined from at least eight independent experiments.

Growth curves

Overnight standing cultures in Luria broth supplemented with thymine (50 μg/ml) and chloramphenicol (20 μg/ml), kanamycin (50 μg/ml), or spectinomycin (20 μg/ml) were diluted 1:1000 into prewarmed fresh medium and shaken at 37°C or at 30°C. Readings were taken every 30 min after the cultures reached 20 Klett units (No. 42 green filter; 5 x 10^7 cells/ml).

Primer extensions

Cultures were grown to approximately 1.25 x 10^8 cells/ml in Luria broth supplemented with thymine (50 μg/ml). At this point the cultures were split, and half of each one was shifted to 42°C for 20 min. Total steady-state RNA was isolated and used for prime extension as described in Arraiano *et al*. (1993) employing 7.5 and 15 μg of RNA along with either primer QLrneL2 or QLrneLM2. Primer extension products were separated on 6% polyacrylamide/7M urea gels. The sequencing ladder for the analysis of the products was obtained using the *fmol* DNA Cycle Sequencing System (Promega) with the same primer.

Western analysis

Western blot analysis was done as described in Ow *et al*. (2000) using 50 μg of total protein lysate and analyzed by 6% SDS/PAGE. The protein gel was electrotransferred to an Immobilon-P transfer membrane (Millipore). The RNase E protein was detected using an RNase E MAP antibody (1:2000) raised against the first 20 amino acid of the N-

terminus (Ow *et al*., 2000). The UvrD protein was visualized using a UvrD antibody at 1:4000 dilution (Washburn and Kushner, 1993). Stripping of the Western blots was done as specified by the membrane's manufacturer. Quantification of band densities was done by scanning the blots on a Stratagene Eagle Eye II densitometer.

Northern analysis

RNA isolation, Northern blot analysis, and half-life determinations were done as described by O'Hara *et al*. (1995).

ACKNOWLEDGEMENTS

We thank R.W. Simons for providing pRS551, λRS45, and λRS74 and for helpful advice on lambda phage biology; S. Bundy for technical assistance; and B. Mohanty and T. Perwez for critical reading of the manuscript. This work was supported in part by NIH grants (GM28760 and GM52210) to S. R. K. M. C. O. was supported by a National Institutes of Health predoctoral training grant (GM07103) for a portion of this work.

REFERENCES

Apirion, D., and A. B. Lassar. 1978. A conditional lethal mutant of *Escherichia coli* which affects the processing of ribosomal RNA. J. Biol. Chem. 253: 1738-1742.

Arraiano, C. M., S. D. Yancey, and S. R. Kushner. 1988. Stabilization of discrete mRNA breakdown products in *ams pnp rnb* multiple mutants of *Escherichia coli* K-12. J. Bacteriol. 170: 4625-4633.

Arraiano, C. M., S. D. Yancey, and S. R. Kushner. 1993. Identification of endonucleolytic cleavage sites involved in decay of *Escherichia coli trxA* mRNA. J. Bacteriol. 175: 1043-1052.

Babitzke, P., and S. R. Kushner. 1991. The *ams* (altered mRNA stability) protein and ribonuclease E are encoded by the same structural gene of *Escherichia coli*. Proc. Natl. Acad. Sci. USA. 88: 1-5.

Blum, E., B. Py, A. J. Carpousis, and C. F. Higgins. 1997. Polyphosphate kinase is a component of the *Escherichia coli* RNA degradosome. Mol. Microbiol. 26: 387-398.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Casadaban, M. J., and S. N. Cohen. 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. 138: 179-207.

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

Claverie-Martin, F., M. R. Diaz-Torres, S. D. Yancey, and S. R. Kushner. 1989. Cloning of the altered mRNA stability (*ams*) gene of *Escherichia coli* K-12. J. Bacteriol. 171: 5479-5486.

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.

Diwa, A., A. L. Bricker, C. Jain, and J. G. Belasco. 2000. An evolutionarily conserved RNA stem-loop functions as a sensor that directs feedback regulation of RNase E gene expression. Genes Dev.14: 1249-1260.

Hajnsdorf, E., F. Braun, J. Haugel-Nielsen, J. Le Derout, and P. Régnier. 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.

Henry, M. D., S. D. Yancey, and S. R. Kushner. 1992. Role of the heat shock response in stability of mRNA in *Escherichia coli* K-12. J. Bacteriol. 174: 743-748.

Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77: 51-59.

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.

Jiang, X., A. Diwa, A., and J. G. Belasco. 2000. Regions of RNase E important for 5'-end dependent RNA cleavage and autoregulated synthesis. J. Bacteriol. 182: 2468-2475.

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

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

López, P. J., I. Marchand, S. A. Joyce, and M. Dreyfus. 1999. The C-terminal half of RNase E, which organizes the *Escherichia coli* degradosome, participates in mRNA degradation but not rRNA processing *in vivo*. Mol. Microbiol. 33: 188-189.

Mackie, G. A. 1991. Specific endonucleolytic cleavage of the mRNA for ribosomal protein S20 of *Escherichia coli* requires the product of the *ams* gene *in vivo* and *in vitro*. J. Bacteriol. 173: 2488-2497.

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

Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

Mohanty, B. K., and S. R. Kushner. 1999b. Residual polyadenylation of poly (A) polymerase I (*pcnB*) mutants of *Escherichia coli* does not result from activity encoded by the *f310* gene. Mol. Microbiol. 34: 1109-1119.

Mudd, E. A., and C. F. Higgins. 1993. *Escherichia coli* endoribonuclease RNase E: autoregulation of expression and site specific cleavage of mRNA. Mol. Microbiol. 9: 557-568.

Mulligan, M. E., D. K. Hawley, R. Entriken, and W. R. McClure. 1984. *Escherichia coli* promoter sequences predict *in vitro* RNA polymerase selectivity. Nucleic Acids Res. 12: 789-800

O'Hara, E. B., J. A. Chekanova, C. A. Ingle, Z. R. Kushner, E. Peters, and S. R. Kushner. 1995. Polyadenylation helps regulate mRNA decay in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 92: 1807-1811.

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

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., H. Cauton, E. A. Mudd, and C. F. Higgins. 1994. A protein complex mediating mRNA degradation in *Escherichia coli*. Mol. Microbiol. 14: 717-729.

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.

Reece, K. S., and G. J. Phillips. 1995. New plasmids carrying antibiotic-resistance cassettes. Gene 165: 141-142.

Régnier, P., and C. M. Arraiano. 2000. Degradation of mRNA in bacteria: emergence of ubiquitous features. Bioessays 22: 235-244.

Silhavy, T. J., M. L. Berman, and L. W. Enquist. 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Simons, R. W., F. Houman, and N. Kleckner. 1987. Improved single and multicopy *lac* based cloning vectors for protein and operon fusions. Gene 53: 85-96.

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

Wang, R. F., S. R. and Kushner. 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and expression in *Escherichia coli*. Gene 100:195-199.

Washburn, B. K., and S. R. Kushner. 1993. Characterization of DNA helicase II from a *uvrD252* mutant of *Escherichia coli*. J. Bacteriol. 175: 341-350.

Table 4.1. Bacterial strains, phages, and plasmids used in this study.

Strain	Genotype	Source or reference
MC1061	F$^-$ *araD139 Δ(ara-leu)7697 ΔlacX74 galE15 galK16 hsdR2 rpsL150*	Casadaban and Cohen (1980)
MG1693	*thyA715 rph-1*	*E. coli* Genetic Stock Center
NK7049	*ΔlacX74 galOP308 rpsL*	Simons *et al*. (1987)
SK5665	*thyA715 rph-1 rne-1*	Arraiano *et al*. (1988)
SK9701	*ΔlacX74 galOP308 rpsL* λMOK2 (p2-*lacZ*)	This study
SK9702	*ΔlacX74 galOP308 rpsL* λMOK3 (p3-*lacZ*)	This study
SK9703	*ΔlacX74 galOP308 rpsL* λMOK4 (p1p2p3-*lacZ*)	This study
SK9704	*ΔlacX74 galOP308 rpsL* λMOK5 (p0-*lacZ*)	This study
SK9705	*rneΔ1018::bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pQLK26 (*rne*$^+$ Km^r)	Ow *et al*. (2000)
SK9714	*rneΔ1018::bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pSBK1 (*rne*$^+$ Cm^r)	Ow *et al*. (2000)
SK9726	*ΔlacX74 galOP308 rpsL150* λMOK1 (p1-*lacZ*)	This study

SK9738	F$^-$ *araD139 Δ(ara-leu)7697 ΔlacX74 galE15 galK16 hsdR2 rpsL150*/ pMOK5 (p1-*lacZ* Km^r)	This study
SK9742	F$^-$ *araD139 Δ(ara-leu)7697 ΔlacX74 galE15 galK16 hsdR2 rpsL150*/ pMOK9 (p2-*lacZ* Km^r)	This study
SK9743	F$^-$ *araD139 Δ(ara-leu)7697 ΔlacX74 galE15 galK16 hsdR2 rpsL150*/ pMOK10 (p3-*lacZ* Km^r)	This study
SK9744	F$^-$ *araD139 Δ(ara-leu)7697 ΔlacX74 galE15 galK16 hsdR2 rpsL150*/ pMOK11 (p1p2p3-*lacZ* Km^r)	This study
SK9745	F$^-$ *araD139 Δ(ara-leu)7697 ΔlacX74 galE15 galK16 hsdR2 rpsL150*/ pMOK12 (p0-*lacZ* Km^r)	This study
SK9750	*rneΔ208 thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r	This study
SK9934	*rneΔ208 thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pSBK1 (*rne*$^+$ 6-8 copy Cm^r)	This study
SK9937	*rneΔ1018::bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pMOK13 (*rne-1* Cm^r)	Ow *et al*. (2000)
SK9946	*rneΔ208 thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pSBK6 (*rneΔp1Δp2Δp3* Cm^r)	This study
SK9993	*rneΔ1018::bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/	This study

	pMOK22 (*rneΔp2* Km^r)	
SK9997	*rneΔ1018::bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pMOK25 (*rneΔp2Δp3* Km^r)	This study
SK10101	*rneΔ1018::bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pMOK27 (*rneΔp3* Km^r)	This study
SK10102	*rneΔ1018::bla thyA715 rph1 recA56 srl300::*Tn10 Tc^r/ pMOK28 (*rneΔp1Δp3* Km^r)	This study
SK10108	*rneΔ1018::bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pMOK30 (*rneΔp1Δp2* Km^r)	This study
SK10109	*rneΔ208 thyA715 rph-1 recA56 srl300::*Tn10 Tc^r/ pMOK30 (*rneΔp1Δp2* Km^r)	This study
SK10110	*rneΔ208 thyA715 rph-1 recA56 srl300::*Tn10 Tc^r/ pMOK28 (*rneΔp1Δp3* Km^r)	This study
SK10111	*rneΔ208 thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pMOK25 (*rneΔp2Δp3* Km^r)	This study
SK10142	*rneΔ208 thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pWSK129 (Km^r) pMOK43 (*rneΔp2Δp3* Sp^r)	This study
SK10143	*rneΔ208 thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/	This study

	pWSK129 (Km^r) pMOK44 (*rne*$^+$ Sp^r)	
SK10144	*rneΔ1018::bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pWSK129 (Km^r) pMOK45 (*rne-1* Sp^r)	This study
SK10145	*rneΔ1018::bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pWSK129 (Km^r) pMOK42 (*rneΔp1Δp3* Sp^r)	This study
SK10146	*rneΔ1018::bla thyA715 rph-1 recA56 srl-300::*Tn*10* Tc^r/ pWSK129 (Km^r) pMOK41 (*rneΔp1Δp2* Sp^r)	This study

Phage	Genotype	Source or reference
λMOK1	Same as λRS45 but containing the p1-*lacZ* transcriptional fusion	This study
λMOK2	Same as λRS45 but containing the p2-*lacZ* transcriptional fusion	This study
λMOK3	Same as λRS45 but containing the p3-*lacZ* transcriptional fusion	This study
λMOK4	Same as λRS45 but containing the p1p2p3-*lacZ* transcriptional fusion	This study
λMOK5	Same as λRS74 but containing the p0-*lacZ* fusion	This study
λRS45	*bla*'-*lacZ*$_{sc}$ *imm21 ind*$^+$	Simons *et al*. (1987)
λRS74	*plac*UV5-*lacZ*$^+$ *imm21 ind*$^+$	Simons *et al*. (1987)

Plasmid	Genotype	Source or reference
pMOK5[a]	Same as pRS551 but with p1-*lacZ* Km^r	This study
pMOK9[a]	Same as pRS551 but with p2-*lacZ* Km^r	This study
pMOK10[a]	Same as pRS551 but with p3-*lacZ* Km^r	This study
pMOK11[a]	Same as pRS551 but with p1p2p3-*lacZ* Km^r	This study
pMOK12[a]	Same as pRS551 but with p0-*lacZ* Km^r	This study
pMOK13[b]	*rne-1* Cm^r	Ow *et al*. (2000)
pMOK22[b]	*rneΔp2* Km^r	This study
pMOK25[b]	*rneΔp2Δp3* Km^r	This study
pMOK27[b]	*rneΔp3* Km^r	This study
pMOK28[b]	*rneΔp1Δp3* Km^r	This study
pMOK30[b]	*rneΔp1Δp2* Km^r	This study
pMOK35[c]	*rneΔp1Δp2* Ap^r	This study
pMOK36[c]	*rneΔp1Δp3* Ap^r	This study
pMOK37[c]	*rneΔp2Δp3* Ap^r	This study

pMOK38 [c]	*rne*$^{+}$ Ap^{r}	This study
pMOK39 [c]	*rne-1* Ap^{r}	This study
pMOK40 [c]	Sp^{r}	This study
pMOK41 [c]	*rneΔp1Δp2* Sp^{r}	This study
pMOK42 [c]	*rneΔp1Δp3* Sp^{r}	This study
pMOK43 [c]	*rneΔp2Δp3* Sp^{r}	This study
pMOK44 [c]	*rne*$^{+}$ Sp^{r}	This study
pMOK45 [c]	*rne-1* Sp^{r}	This study
pQLK26 [b]	*rne*$^{+}$ Km^{r}	Ow *et al*. (2000)
pRS551 [a]	Km^{r}	Simons *et al*., (1987)
pSBK1 [b]	*rne*$^{+}$ Cm^{r}	Ow *et al*. (2000)
pSBK6 [b]	*rneΔp1Δp2Δp3* Cm^{r}	This study
pSBK29 [b]	Cm^{r}	Mohanty and Kushner (1999)
pVMK94 [c]	Ap^{r}	This study
pWSK129 [b]	6-8 copy plasmid Km^{r}	Wang and Kushner (1991)

[a] ColE1 multi-copy origin of DNA replication.

[b] pSC101 6-8 copy number origin of DNA replication.

[c] mini-F single copy origin of DNA replication.

Table 4.2. mRNA decay rates of *rpsT* and *rpsO* at 37°C in strains carrying 6-8 copies of the *rne* promoter constructs.

Transcript	Half-life (min)[a]			
	rne$^+$	*rneΔp2Δp3*	*rneΔp1Δp3*	*rneΔp1Δp2*
rpsO[b]	1.5 ± 0.4	1.7 ± 0.2	1.9 ± 0.1	2.3 ± 0.4
rpsT[c]	1.4 ± 0.3	1.4 ± 0.3	1.7 ± 0.2	2.1 ± 0.2

[a] Half-lives were determined as described in Experimental Procedures and represent the average of three independent determinations.

[b] The data presented is for the *rpsO2* transcript, which is generated by RNase E cleavage at the 3' end of the primary transcript (Hajnsdorf *et al*., 1996; Ow *et al*., 2000).

[c] For *rpsT* the data from both full-length transcripts derived from p1 and p2 (Mackie, 1991) were averaged.

Table 4.3. mRNA decay rates of *rpsT* and *rpsO* at 37°C or 30°C in strains with single copy *rne* promoter constructs.

(A) 37°C

Transcript	Half-life (min)[a]			
	rne$^+$	*rneΔp2Δp3*	*rneΔp1Δp3*	*rneΔp1Δp2*
rpsO	2.3 ± 0.3	3.2 ± 0.5	5.1 ± 0.5	3.4 ± 0.3
rpsT	2.0 ± 0.4	3.2 ± 0.5	3.1 ± 0.7	2.7 ± 0.5

(B) 30°C

Transcript	Half-life (min)[a]			
	rne$^+$	*rneΔp2Δp3*	*rneΔp1Δp3*	*rneΔp1Δp2*
rpsO	6.3 ± 1.2	14.2 ± 2.4	> 30.0	13.5 ± 1.3
rpsT	4.0 ± 0.7	6.3 ± 0.9	12.4 ± 2.4	6.3 ± 0.3

[a]Half-lives were determined as described in Experimental Procedures and represent the average of three independent determinations. Data are presented as described in Table 4.2.

Table 4.4. Primers used in this study.

Primer	Sequence
QLrneL2	5' ccatctaccggtaaggactgc 3'
QLrneLM2	5' ggtcgggcaaaatgggttattcc 3'
mornep1a	5' gc**gaattc**gtaagcgggtgataaatgg 3'
mornep1b	5' ta**ggatcc**agatcaatacgtcttacgc 3'
mornep2a	5' at**gaattc**taccttattacttcactgcg 3'
mornep2b-1	5' tt**ggatcc**ctgcaagccttgttatagc 3'
mornep3a-1	5' ta**gaattc**gattctaattagccaacagg 3'
mornep3b-1	5' tg**ggatcc**acttcttgctggtgattgg 3'
mornep3a	5' gc**gaattc**attcgtcagcggtgatagc 3'
prneX	5' tgggatccaatgagtggcgcgaatcc 3'
morne4	5' tgtgggattaactgcgcgtcg 3'
molacZ5'	5' cacgacgttgtaaaacgacg 3'
RNE-1014	5' cggtgctt**gaattc**tcttcacatgcgactg 3'
RNE+3628	5' cat**tctaga**ttagcaaggatgccattc 3'
RNEP1ARMB	5' caaggatggacacggaaacggacttttaccatttatcacc 3'
RNEP1ARMC	5' ggtgataaatggtaaaagtccgtttccgtgtccatccttg 3'
RNEP2ARMB	5' caaggatggacacggaaacgcacttcttgctggtgattgg 3'
RNEP2ARMC	5' ccaatcaccagcaagaagtgtcatcttgctataacaaggct 3'
RNEP3ARMB	5' gcagtgaagtaataaggtgctaattagaatcgccaaccat 3'
RNEP3ARMC	5' atggttggcgattctaattagcaccttattacttcactgc 3'

P1P2ARMB	5' caaggatggacacggaaacgcacttcttgctggtgattgg 3'
P1P2ARMC	5' ccaatcaccagcaagaagtgcgtttccgtgtccatccttg 3'

In bold are the engineered restriction sites for *Eco*RI (5' gaattc 3'), *Bam*HI (5' ggatcc 3'), and *Xba*I (5' tctaga 3').

Figure 4.1. Primer extension analysis of the *rne* 5' UTR. Total RNA (7.5 or 15 μg) was isolated from MG1693 (*rne*$^+$; lanes 1-2, 5-6) and SK5665 (*rne-1*; lanes 3-4, 7-8) at 30ºC or 20 minutes after shift to 42ºC (lanes 1-4). G, A, T, C comprise the sequencing ladder of pQLK26 using primer QLrneL2. The original transcription start-site of the *rne* gene is indicated as p1 (Claverie-Martin *et al.,* 1991) and the additional promoter start sites are marked as p2 and p3. The RNase E cleavage sites are indicated by the brackets. Similar results were obtained using a second primer, QLrneLM2.

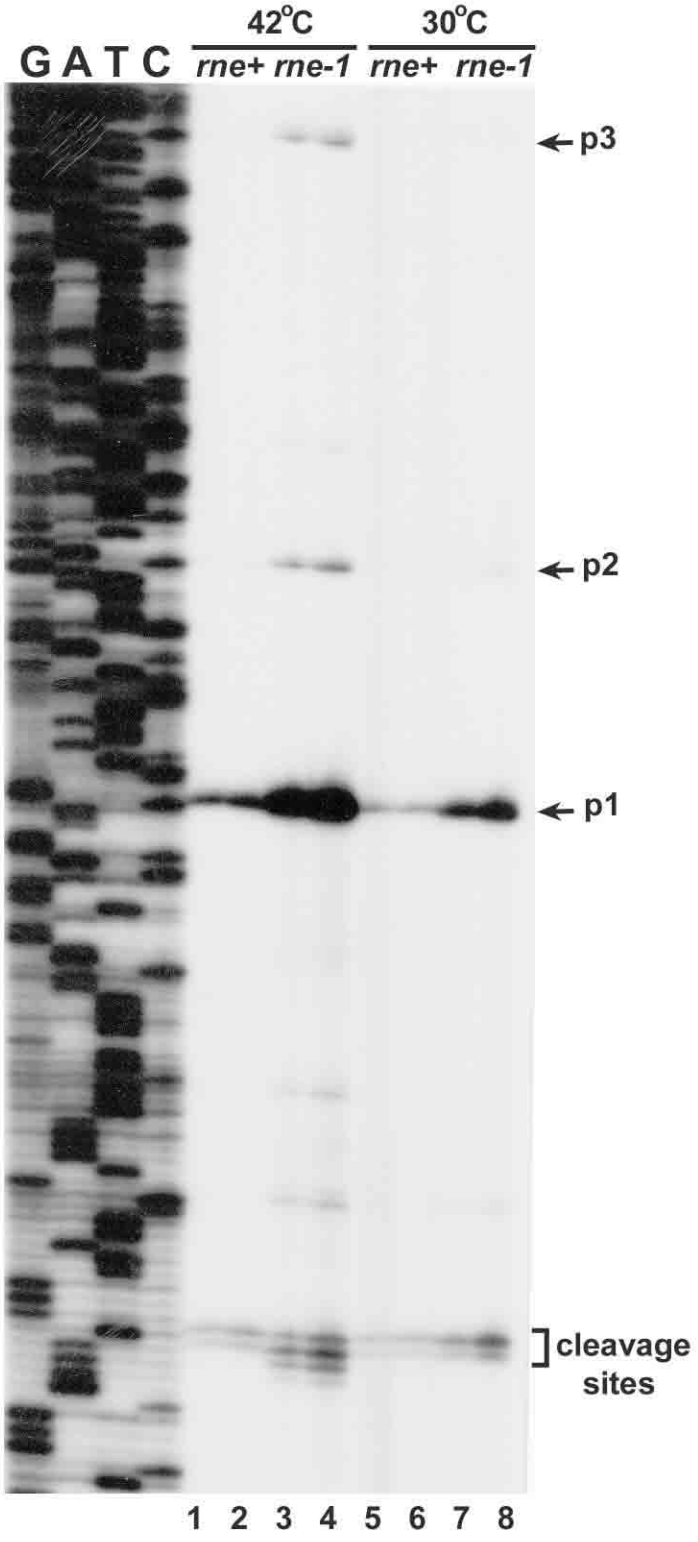

42°C
30°C
G A T C
rne+ rne-1 rne+ rne-1
p3
p2
p1
cleavage sites
1 2 3 4 5 6 7 8

Figure 4.2. The *rne* promoter region. The transcriptional start sites for p1, p2, and p3 are shown by the arrows. The -10 and -35 hexamers for each promoter are boxed. Numbering was assigned using the transcriptional start site of p1 (+1) as the reference point.

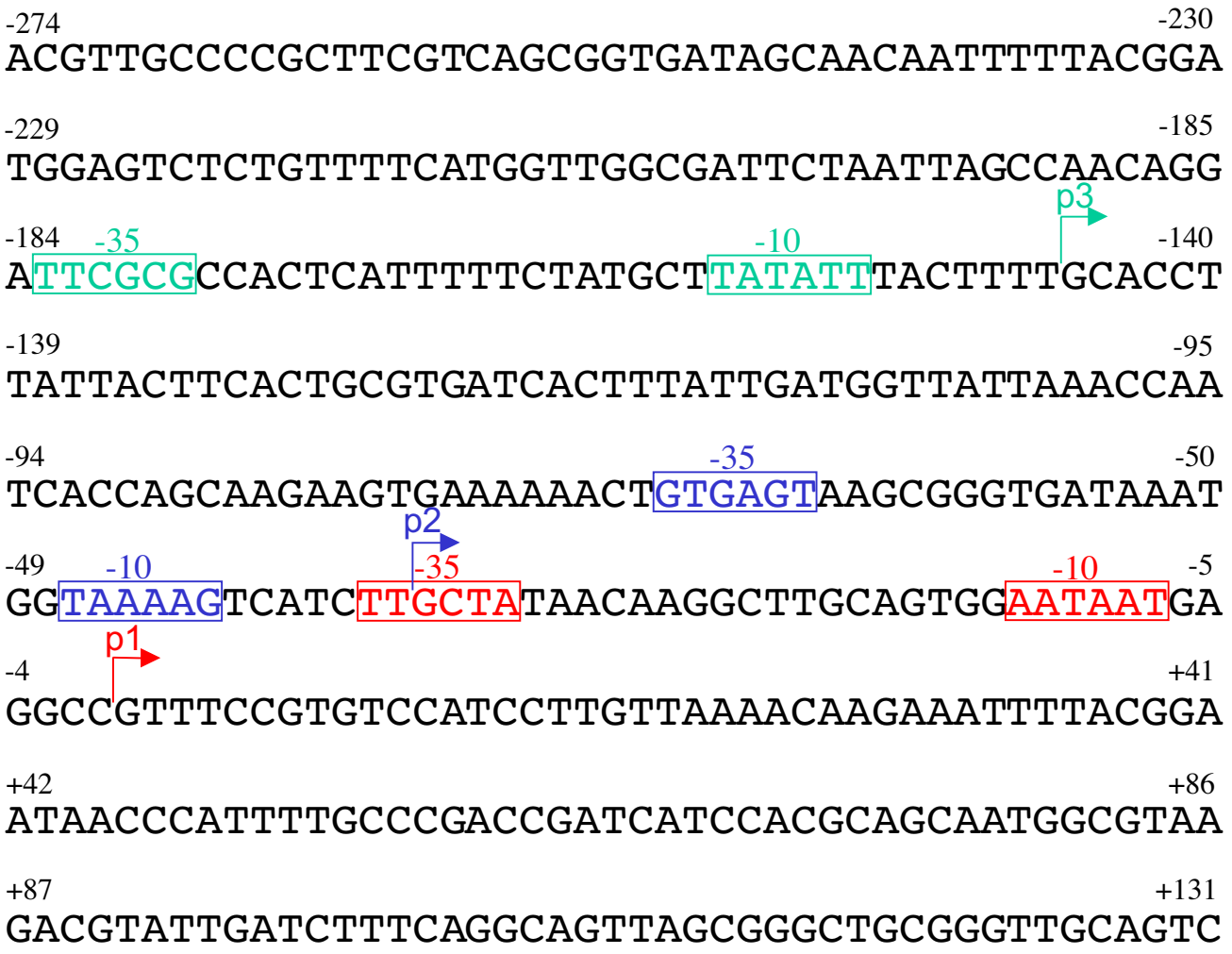

-274
-230
ACGTTGCCCCGCTTCGTCAGCGGTGATAGCAACAATTTTTACGGA
-229
-185
TGGAGTCTCTGTTTTCATGGTTGGCGATTCTAATTAGCCAACAGG
p3
-184
-35
-10
-140
ATTCGCGCCACTCATTTTTCTATGCTTATATTTACTTTTGCACCT
-139
-95
TATTACTTCACTGCGTGATCACTTTATTGATGGTTATTAAACCAA
-94
-35
-50
TCACCAGCAAGAAGTGAAAAAACTGTGAGTAAGCGGGTGATAAAT
p2
-49
-10
-35
-10
-5
GGTAAAAGTCATCTTGCTATAACAAGGCTTGCAGTGGAATAATGA
p1
-4
+41
GGCCGTTTCCGTGTCCATCCTTGTTAAAACAAGAAATTTTACGGA
+42
+86
ATAACCCATTTTGCCCGACCGATCATCCACGCAGCAATGGCGTAA
+87
+131
GACGTATTGATCTTTCAGGCAGTTAGCGGGCTGCGGGTTGCAGTC

Figure 4.3. Transcriptional activity of the various *rne* promoters. Aliquots (0-125 μg) of total protein extracts obtained from lysogen strains SK9726 (□; *ΔlacX74 galOP308 rpsL150* λMOK1 [p1-*lacZ*]), SK9701 (◊; *ΔlacX74 galOP308 rpsL150* λMOK2 [p2-*lacZ*]), SK9702 (○; *ΔlacX74 galOP308 rpsL150* λMOK3 [p3-*lacZ*]), SK9703 (△; *ΔlacX74 galOP308 rpsL150* λMOK4 [p1p2p3-*lacZ*]), and SK9704 (⊞; *ΔlacX74 galOP308 rpsL150* λMOK5 [p0-*lacZ*]) were used to analyze ß-galactosidase activity as described in Experimental Procedures. The data presented are averages of at least eight independent determinations.

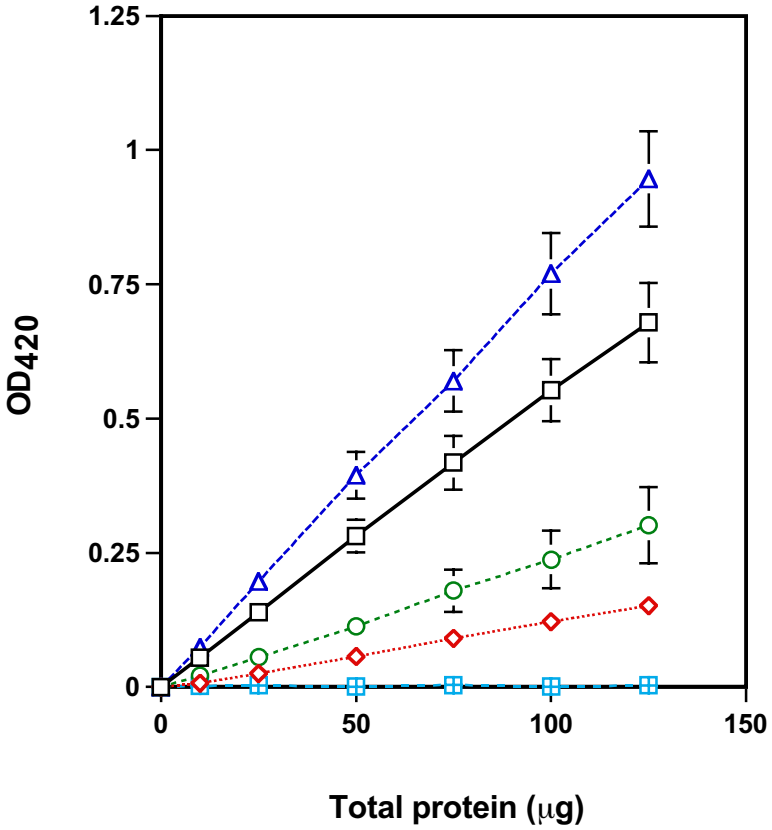

1.25
1
0.75
0.5
0.25
0
0
50
100
150
OD420
Total protein (μg)

Figure 4.4. Schematic of the *rne* promoter deletions. Shown at the top is the wild-type region with the transcriptional start sites for the p1, p2, and p3 promoters. All promoter deletion alleles carry the entire coding region of *rne*. The *rneΔ208* allele is a chromosomal deletion of 294 nt of the 5' UTR between nucleotides +10 and + 304. Numbering for nucleotides is as shown in Figure 4.2. All deletions were confirmed by DNA sequencing. The locations of the two primers, QLrneL2 and QlrneLM2, used for the primer extension experiments are indicated by the hatched rectangles. Diagram is not drawn to scale.

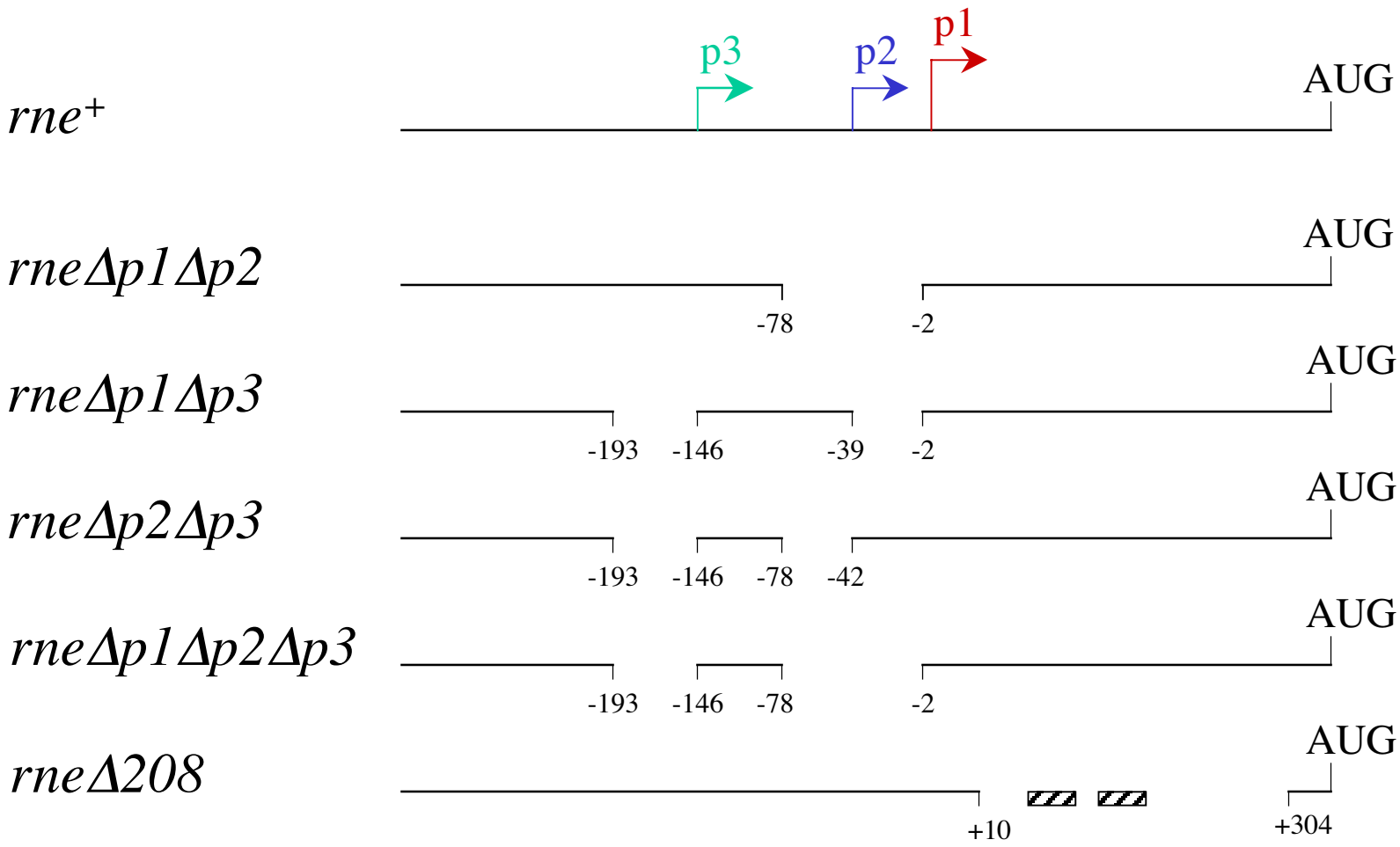

rne+
p3
p2
p1
AUG
rneΔp1Δp2
-78
-2
AUG
rneΔp1Δp3
-193
-146
-39
-2
AUG
rneΔp2Δp3
-193
-146
-78
-42
AUG
rneΔp1Δp2Δp3
-193
-146
-78
-2
AUG
rneΔ208
+10
+304
AUG

Figure 4.5. RNase E Western analysis of *rne* promoter deletion mutants at 37ºC. Fifty μg of each total protein lysate was electrophoresed on a 6% SDS/PAGE gel and electrotransferred to a PVDF (polyvinylidene fluoride) membrane (Immobilon-P; Millipore). Detection of the RNase E protein (top panel) was done by probing with an RNase E MAP antibody (Ow *et al*., 2000). The RNase E Westerns were subsequently stripped and reprobed with a UvrD antibody (Washburn and Kushner, 1993) which served as a normalization control (bottom panel). Low-copy (L) and single-copy (S) refer to the copy number of the resident plasmid in the *rneΔ1018::bla* host strain. Lane 1, SK9714 (*rneΔ1018::bla*/ pSBK1 [*rne*$^+$ Cm^r]); lane 2, SK10143 (*rneΔ1018::bla*/ pMOK44 [*rne*$^+$ Sp^r]); lane 3, SK10108 (*rneΔ1018::bla*/ pMOK30 [*rneΔp1Δp2* Km^r]); lane 4, SK10146 (*rneΔ1018::bla*/ pMOK41 [*rneΔp1Δp2* Sp^r]); lane 5, SK10102 (*rneΔ1018::bla*/ pMOK28 [*rneΔp1Δp3* Km^r]); lane 6, SK10145 (*rneΔ1018::bla*/ pMOK42 [*rneΔp1Δp3* Sp^r]); lane 7, SK9997 (*rneΔ1018::bla*/ pMOK25 [*rneΔp2Δp3* Km^r]); lane 8, SK10142 (*rneΔ1018::bla*/ pMOK43 [*rneΔp2Δp3* Sp^r]). RQ, relative quantity, was determined by quantification of both the RNase E and UvrD bands using a Stratagene Eagle II Densitometer. The protein levels for RNase E and UvrD for SK10143 (*rneΔ1018::bla*/ pMOK44 [*rne*$^+$ Sp^r]) was set at one.

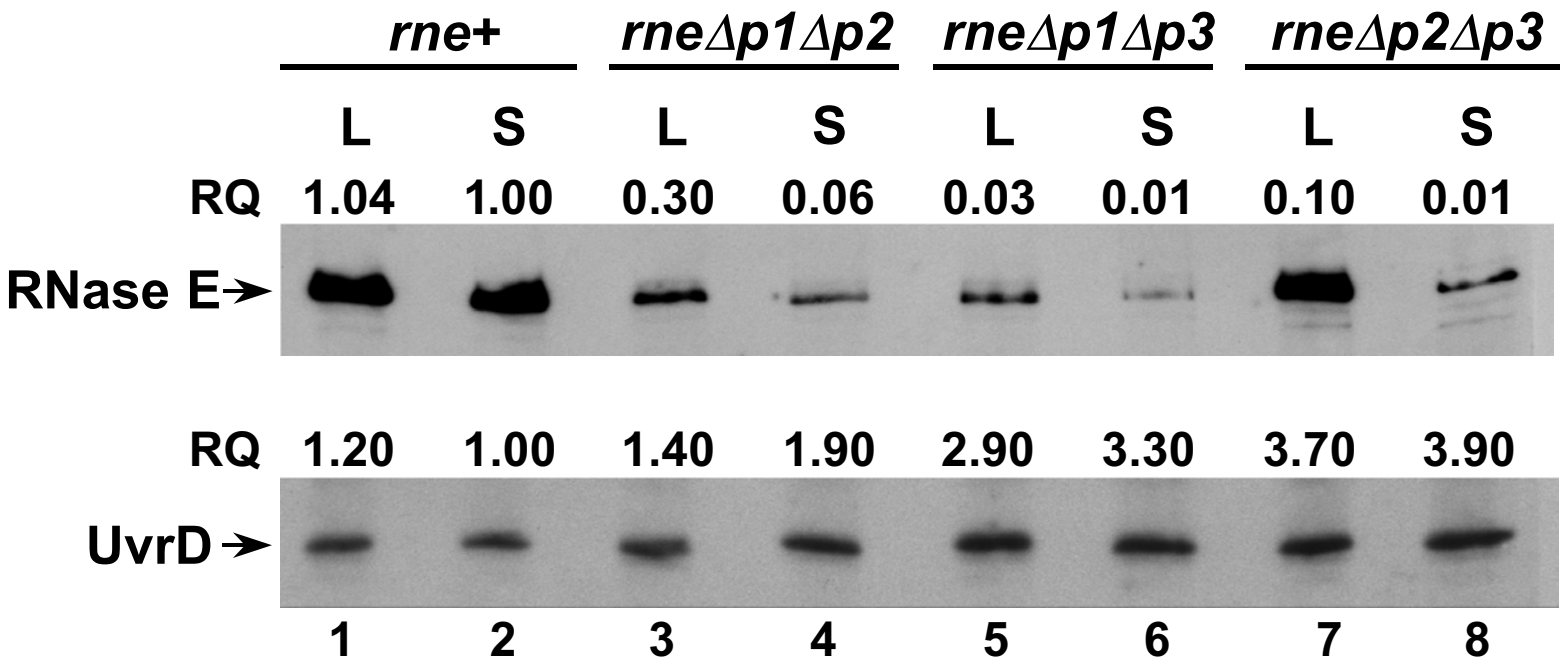

rne+
rneΔp1Δp2
rneΔp1Δp3
rneΔp2Δp3
L
S
L
S
L
S
L
S
RQ 1.04 1.00 0.30 0.06 0.03 0.01 0.10 0.01
RNase E
RQ 1.20 1.00 1.40 1.90 2.90 3.30 3.70 3.90
UvrD
1 2 3 4 5 6 7 8

Figure 4.6. Decay profile of the *rpsO* mRNA at 30°C. Northern blot analysis and total RNA isolation were done as described in Experimental Procedures on strains SK10143 (*rneΔ1018::bla*/ pMOK44 [*rne*$^{+}$ Sp^{r}]), SK10146 (*rneΔ1018::bla*/ pMOK41 [*rneΔp1Δp2* Sp^{r}]), SK10145 (*rneΔ1018::bla*/ pMOK42 [*rneΔp1Δp3* Sp^{r}]), and SK10142 (*rneΔ1018::bla*/ pMOK43 [*rneΔp2Δp3* Sp^{r}]). Time points in minutes were taken after rifampicin addition. Equivalent amounts of RNA (5 μg) were loaded into each lane of a 6% polyacrylamide/7 M urea gel. The *rpsO* mRNA shown here is the transcript generated from an RNase E cleavage at the 3' end of the *rpsO* primary transcript (*rpsO2*) (Hajnsdorf *et al*., 1996; Ow *et al*., 2000).

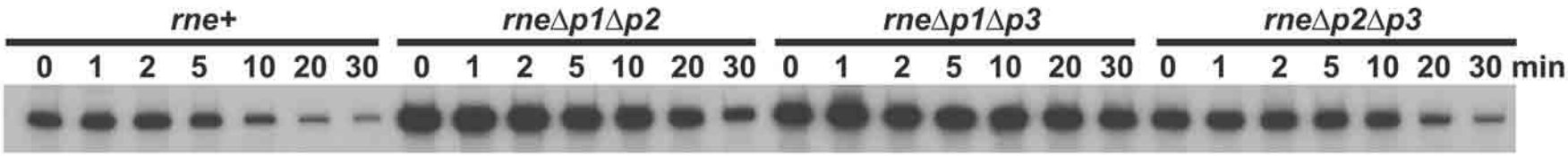
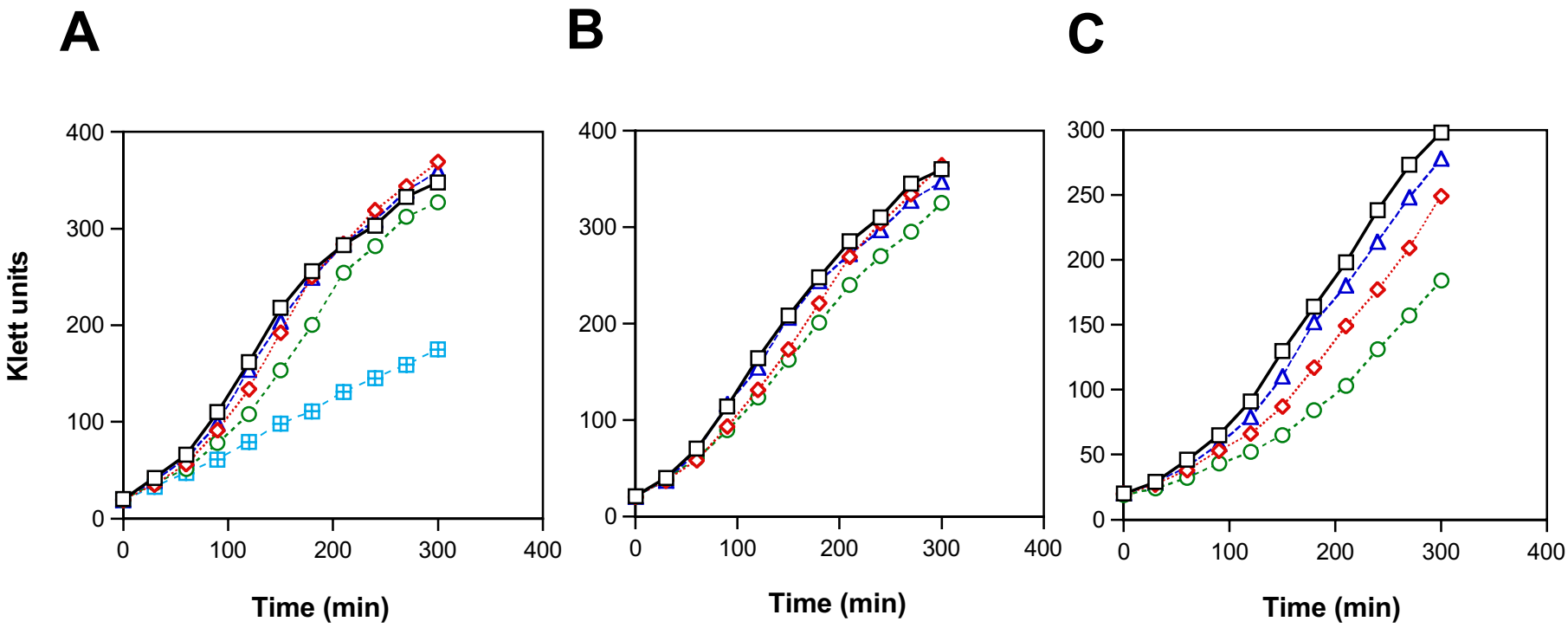

rne+
rneΔp1Δp2
rneΔp1Δp3
rneΔp2Δp3
0 1 2 5 10 20 30 0 1 2 5 10 20 30 0 1 2 5 10 20 30 0 1 2 5 10 20 30 min

Figure 4.7. Growth properties of promoter deletion strains. Overnight standing cultures of SK10143 (*rneΔ1018::bla*/ pMOK44 [*rne*$^{+}$ Sp^{r}]), SK10146 (*rneΔ1018::bla*/ pMOK41 [*rneΔp1Δp2* Sp^{r}]), SK10145 (*rneΔ1018::bla*/ pMOK42 [*rneΔp1Δp3* Sp^{r}]), SK10142 (*rneΔ1018::bla*/ pMOK43 [*rneΔp2Δp3* Sp^{r}]), and SK10144 (*rneΔ1018::bla*/ pMOK45 [*rne-1* Sp^{r}]) in Luria broth with thymine (50 μg/ml) and spectinomycin (20 μg/ml) were diluted 1:1000 with fresh prewarmed medium. Cultures were shaken at 37^{o}C (B), or 30^{o}C (C) until they reached Klett 20 (5 x 10^{7} cells/ml) after which time Klett readings were taken every 30 minutes. For the 44^{o}C growth curve (A), the cultures were grown at 37^{o}C until they reached Klett 20, then shifted to 44^{o}C, and readings were taken every 30 minutes. Generation times at 37^{o}C were 30, 34, 33, and 33 minutes for SK10143, SK10146, SK1045, and SK10142, respectively. Generation times at 30^{o}C for SK10143, SK1046, SK10145, SK10142 were 58, 68, 86, and 61 minutes, respectively. Generation times at 44^{o}C for SK10143, SK10146, SK10145, and SK10142 were 45, 48, 50, and 57 min, respectively. □, SK10143; ◊, SK10146; ○, SK10145; △, SK10142; ⊞, SK10144.


A
B
C
Klett units
Time (min)
Time (min)
Time (min)
0
100
200
300
400
50
150
250

CHAPTER 5

CONCLUSIONS

CONCLUSIONS

It has become increasingly clear that the rate at which an mRNA decays is an important parameter in the control of its expression. By varying the longevity of mRNAs in response to environmental changes, prokaryotic organisms can adjust their metabolic processes so as to improve their survival rate. Because issues regarding mRNA turnover addressed in *Escherichia coli* are likely to find parallels in other prokaryotic systems, studies in *E. coli* have been instrumental in unraveling the pathways of mRNA turnover. The observations that individual messages in *E. coli* had a specific half-life regardless of their length (Hirashima *et al*., 1973; von Gabain *et al*., 1983; Arraiano *et al*., 1993) strongly indicated that the turnover of transcripts was not a contingent process but that their decay was controlled throughout the lifetime of a cell by finely tuned mechanisms (Kushner, 1996). The identification of *cis*- (*e.g*., RNA stem-loop structures) and *trans*-acting factors (*e.g*., ribonucleases) involved in this important *E. coli* cellular process has also helped in the understanding of how mRNA degradation occurs and how it is regulated.

The oldest recognized *cis*-acting factors are stem-loop structures situated ubiquitously throughout an RNA molecule. By affecting the processivity of 3' to 5' exoribonucleases, these motifs directly influence the turnover rate of an RNA (Coburn and Mackie, 1999; Régnier and Arraiano, 2000; Steege, 2000). In recent years, other factors present at the 5' termini (single-strandedness and phosphorylation state) as well as the 3' termini (polyadenylation) of an RNA have also been shown to participate in modulating the turnover rate of mRNAs (O'Hara *et al*., 1995; Mackie, 1998; Mohanty and Kushner, 1999; Mackie, 2000; Spickler *et al*., 2001).

One of the most important *trans*-acting factors regulating mRNA degradation is RNase E. Mutations in its gene, *rne*, result in the slowdown of the decay of total pulse-labeled RNA and of specific transcripts (Kuwano *et al*., 1977; Ono and Kuwano, 1979; Cohen, 1997). Additional evidence to support the global role of RNase E in mRNA decay comes from DNA macroarray analysis employing the *rneΔ610* allele described in Chapter 2 (Ow *et al*., 2000). At 37^{o}C, the steady-state levels of the vast majority of the 4,200 ORFs in

E. coli are increased significantly over the wild-type strain (Ow and Kushner, unpublished observations). This indicates that the absence of a normal RNase E protein affects the level of almost every transcript in *E. coli*, a characteristic attributable to no other ribonuclease.

In addition, an exciting discovery in the past few years has been the association of RNase E with a 3' to 5' exoribonuclease (PNPase) and an RNA helicase (RhlB) (Carpousis, *et al*., 1994; Py *et al*., 1994; 1996; Kido *et al*., 1996; Miczak *et al*., 1996). This multi-protein apparatus, referred to as the degradosome, consists of all the components that would conceptually be necessary for the degradation of RNAs. Evidence for the *in vivo* existence of the degradosome (Liou *et al*., 2001) along with *in vitro* studies in which reconstituted degradosomes decay transcripts at a similar rate as those *in vivo* (Coburn and Mackie, 1998; Coburn *et al*., 1999) has fostered the notion that this protein complex is an important participant in mRNA turnover. Our study, however, presented in Chapter 2 shows that the relevance of degradosome assembly in mRNA decay is not as clear as previously believed (Ow *et al*., 2000). However, because we observed a slower than normal generation time for strains lacking degradosome assembly (*rneΔ225* and *rneΔ374*; Chapter 2), the degradosome may have some functionality in the cell. If it participates in mRNA turnover it is likely to be involved in the decay of only a small subset of mRNAs. It is also possible that the degradosome is important for *in vivo* mRNA decay only under conditions not employed in our study (*e.g*., different growth stages or stress conditions).

Does the degradosome have a biological function or is its presence solely an artifact of the RNase E purification procedure? The latter is not likely since independent laboratories using different immunological methods have detected the association of the degradosome proteins. Since the C-terminus half of RNase E is dispensible for the function of this protein in mRNA decay and since RNase E cross-reacts with an antibody raised against the yeast myosin (Chapter 2; Casarégola *et al*., 1990; 1992; 1994), the intriguing possibility is raised that this unusually large ribonuclease has additional functions in *E. coli*. What process(es) other than mRNA turnover the *E. coli* degradosome might be involved in, however, remains to be determined. Nevertheless, the observations on the effect

of degradosome disruption on mRNA decay (*rneΔ225* and *rneΔ374*) mean that the current notion of this protein complex as a significant player in mRNA turnover must be reevaluated.

For many years, it was believed that the temperature sensitivity associated with RNase E mutant strains was directly attributed to either their slower than normal mRNA turnover rates or to defects in rRNA processing. This was a logical assumption since the abnormal longevity of mRNAs could lead to the increased production of their protein products (some of which might be deleterious at high levels) or to the sequestration of the translational machinery. In addition, affecting the processing of the 9S and 16S rRNA could affect the proper assembly of ribosomes. However, studies have shown that the assembly of ribosomes can occur in the presence of improperly processed 16S rRNA (Li *et al*., 1999), implying that there is a certain amount of leeway when it comes to assembling the translational machinery.

The results from Chapters 2 and 3 are difficult to reconcile with the idea that defects in mRNA decay or 9S rRNA processing cause the cessation of growth at 44ºC in RNase E conditional lethal mutants. At the 37ºC permissive temperature, mRNAs in *rneΔ610* were stabilized many-fold over the rates obtained in *rne-1* at the nonpermissive temperature without the former incurring any inhibition in growth. Also, the processing of 9S rRNA was not significantly affected in *rneΔ610* at 37ºC or at 44ºC (Ow *et al*., 2000; Chapter 2). These observations strongly suggest that the cessation of growth of RNase E mutants must be caused by the loss of another essential function of RNase E.

One possibility for the essential role of RNase E is the processing of tRNA precursors. Slowing the normal rate of production of tRNAs would eventually lead to a slowdown of translation, followed by starvation, and eventually death. Depleting the tRNA pool could result in fixing the ribosomes on mRNAs and concomitantly preventing ribonucleases from accessing the mRNA for degradation (hence stabilizing the transcript). The isolation of a temperature-resistant revertant, *rneΔ645*, of the *rneΔ610* truncation mutant helped us answer this question. We consistently observed faster processing rates

for the full-length tRNA precursors in the *rneΔ645* revertant as compared to the *rne-1* and *rneΔ610* strains (Chapter 3). It is likely that a consequence of decreasing tRNA levels at the high temperature in *rne-1* or *rneΔ610* is starvation, as witnessed by the growth properties of these mutants. The *rne-1* mutant was more defective in tRNA processing than *rneΔ610*. This phenotypic property correlated directly with the observation that the *rne-1* mutant ceased growing at the high temperature sooner than *rneΔ610*. Are defects in other cellular processes the reason for the inviability of *rne-1* or *rneΔ610* at 44°C? At this moment this possibility cannot be entirely ruled out. Nevertheless, the results from Chapter 3 show that RNase E is involved in all the major aspects of RNA metabolism (mRNA decay, rRNA processing, tRNA processing), surpassing all of the known ribonucleases in *E. coli* in terms of its importance.

For a protein that is so intimately involved in many aspects of RNA metabolism, one would expect that there would be multiple ways to regulate its expression. In fact, many of the ribonucleases implicated in mRNA turnover are regulated (Portier *et al*., 1987; Bardwell *et al*., 1989; Matsunaga *et al*., 1996; Zilhão *et al*., 1996). The identification of two additional promoters for *rne* (Chapter 4) suggests another level of complexity for RNase E. Since all three promoters are needed for normal growth and mRNA decay, each promoter must have some role in adjusting the intracellular level of RNase E. What is most surprising is that the cell carries an excess of RNase E protein, most of which is not involved in degrading mRNAs. The functionality of the extra RNase E protein in the cell may be in other processes aside from RNA metabolism, which were more readily observed in cultures grown at low temperatures. It is also possible that not all of the RNase E protein detected by Western blotting is actually functionally active. It is apparent, however, that the regulation of this endoribonuclease will turn out to be much more complex than previously thought.

RNase E clearly acts on some substrates differently than it does on others. For example, *rneΔ610* has more of an affect on mRNA decay than the *rne-1* allele. The reverse is true for *rne-1*, with 9S rRNA and tRNA processing being more defective with this allele

than with *rneΔ610* (Chapters 2, 3). In addition, the observation that an even shorter version of *rneΔ610*, *rneΔ645*, can partially rescue mRNA decay and tRNA processing means that a further truncation of the protein leads to a more favorable structure so that it can more efficiently act on RNA substrates. Unfortunately, the nature of this conformational change cannot be assessed at this time. To help in this matter, the crystallization of RNase E would be invaluable. Obtaining the crystal structure of this important ribonuclease will not only help in deciphering some of these questions in *E. coli* but also aid in understanding how mechanisms of RNA metabolism function at the most basic level -how a ribonuclease acts on its RNA substrate. In addition, it will provide clues to which other regions of this large protein are important for its function, ultimately leading to the systematic dissection of RNase E.

REFERENCES

Arraiano, C. M., S. D. Yancey, and S. R. Kushner. 1993. Identification of endonucleolytic cleavage sites involved in decay of *Escherichia coli trxA* mRNA. J. Bacteriol. 175: 1043-1052.

Bardwell, J. C. A., P. Régnier, S. M. Chen, Y. Nakamura, M. Grunberg-Manago, and D. L. Court. 1989. Autoregulation of RNase III operon by mRNA processing. EMBO J. 8: 3401-3407.

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

Casarégola, S., V. Norris, M. Goldberg, and I. B. Holland. 1990. Indentification of a 180 kDa protein in *Escherichia coli* related to a yeast heavy-chain myosin. Mol. Microbiol. 4: 505-511.

Casarégola, S., A. Jacq, D. Laoudj, G. McGurk, S. Margarson, M. Tempête, V. Norris, and I. B. Holland. 1992. Cloning and analysis of the entire *Escherichia coli ams*

gene: *ams* is identical to *hmp1* and encodes a 114 kDa protein that migrates as a 180 kDa protein. J. Mol. Biol. 228: 30-40.

Casarégola, S., A. Jacq, D. Laoudj, G. McGurk, S. Margarson, M. Tempête, V. Norris, and I. B. Holland. 1994. Cloning and analysis of the entire *Escherichia coli ams* gene: *ams* is identical to *hmp1* and encodes a 114 kDa protein that migrates as a 180 kDa protein. J. Mol. Biol. 238: 867.

Coburn, G. A., and G. A. Mackie. 1998. Reconstitution of the degradation of the mRNA for ribosomal protein S20 with purified enzymes. J. Mol. Biol. 279: 1061-1074.

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.

Coburn, G. A., X. Miao, D. J. Briant, and G. A. Mackie. 1999. Reconstitution of a minimal degradosome demonstrates functional coordination between a 3' exonuclease and a DEAD-box RNA helicase. Genes Dev. 13: 2594-2603.

Cohen, S. N. 1997. RNase E: still a wonderfully mysterious enzyme. Mol. Microbiol. 23: 1099-1106.

Diwa, A., A. L. Bricker, C. Jain, and J. G. Belasco. 2000. An evolutionarily conserved RNA stem-loop functions as a sensor that directs feedback regulation of RNase E gene expression. Genes Dev. 14: 1249-1260.

Hirashima, A., G. Childs, and M. Inouye. 1973. Differential inhibitory effects of antibiotics on the biosynthesis of envelope proteins of *Escherichia coli*. J. Mol. Biol. 79: 373-389.

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.

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

Kushner, S. R. 1996. mRNA decay. In: Neidhardt, F. C., Curtiss III, R., Ingraham, J. L., Lin, E. C. C., Low, J., Magasanik, K. B. B., Reznikoff, W. S., Riley, M., Schaechter, M., and H.E. Umbarger, eds. *Escherichia coli and Salmonella typhimurium: Cellular and molecular biology*, vol. 2. American Society for Microbiology Press, Washington, D. C. pp. 849-860.

Kuwano, M., M. Ono, H. Endo, K. Hori, K. Nakamura, Y. Hirota, and Y. Ohnishi. 1977. Gene affecting longevity of messenger RNA: a mutant of *Escherichia coli* with altered mRNA stability. Mol. Gen. Genet. 154: 279-285.

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

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

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

Mackie, G. A. 2000. Stabilization of circular *rpsT* mRNA demonstrates the 5'-end dependence of RNase E action *in vivo*. J. Biol. Chem. 275: 25069-25072.

Matsunaga, J., E. L. Simons, and R. W. Simons. 1996. RNase III autoregulation: structure and function of *rncO*, the posttranscriptional "operator." RNA 2: 1228-1240.

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.

Mohanty, B. K., and S. R. Kushner. 1999. Analysis of the function of *Escherichia coli* poly (A) polymerase I in RNA metabolism. Mol. Microbiol. 34: 1094-1108.

O'Hara, E. B., J. A. Chekanova, C. A. Ingle, Z. R. Kushner, E. Peters, and S. R. Kushner. 1995. Polyadenylation helps regulate mRNA decay in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 92: 1807-1811.

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

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.

Portier, C., L. Dondon, M. Grunberg-Manago, and P. Régnier. 1987. The first step in the functional inactivation of the *Escherichia coli* polynucleotide phosphorylase messenger is a ribonuclease III processing at the 5' end. EMBO J. 6: 2165-2170.

Py, B., H. Cauton, E. A. Mudd, and C. F. Higgins. 1994. A protein complex mediating mRNA degradation in *Escherichia coli*. Mol. Microbiol. 14: 717-729.

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.

Régnier, P., and C. M. Arraiano. 2000. Degradation of mRNA in bacteria: emergence of ubiquitous features. Bioessays 22: 235-244.

Spickler, C., V. Stronge, and G. A. Mackie. 2001. Preferential cleavage of degradative intermediates of *rpsT* mRNA by the *Escherichia coli* RNA degradosome. J. Bacteriol. 183: 1106-1109.

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

von Gabain, A., J. G. Belasco, J. L. Schottel, A. C. Y. Chang, and S. N. Cohen. 1983. Decay of mRNA in *Escherichia coli*: investigation of the fate of specific segments of transcripts. Proc. Natl. Acad. Sci. USA 80: 653-657.

Zilhão, R., F. Cairrão, P. Régnier, and C. M. Arraiano. 1996. PNPase modulates RNase II expression in *Escherichia coli*: implications for mRNA decay and cell metabolism. Mol. Microbiol. 20: 1033-1042.