ENDONUCLEASE PROCESSING IN THE RIF REGION
OF THE *ESCHERICHIA COLI* CHROMOSOME

by

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ABSTRACT

Endonuclease processing of mRNA derived from the rif region near 90 minutes on the *Escherichia coli* chromosome was examined. This region contains the *secE-nusG* and the *rplKAJL-rpoBC* (ribosomal protein) operons and it is thought that endonuclease processing by RNase E and RNase III plays some role in translational regulation and/or degradation of these transcripts. The purpose of this study was to investigate the effect that RNase E and RNase III cleavage had on the stability of transcripts derived from the rif region.

Several RNase E and RNase III cleavage sites were identified in the *secE-nusG* and ribosomal protein operons. Total RNA was isolated from wild type and RNase E^{ts} or RNase III deficient *E. coli* strains and hybridized to DNA probes from the rif region. By S1 nuclease protection assays, cleavage by RNase E and RNase III could be designated to those sites which were not cleaved in the RNase E^{ts} (nonpermissive temperature) or RNase III deficient strains. The *secE-nusG* leader region contains an RNase III site and has the potential to form a secondary structure characteristic of other known substrates of RNase III. An RNase E site identified in the L1-L10 intergenic space shares striking sequence and structural similarity with the RNase E consensus sequence and downstream stem loop thought to be involved in RNase E recognition. As well, another processing site was observed in the L1-L10 intergenic space which may be a result of RNase III activity or processing by an unidentified nuclease.

The effect of RNase E and RNase III processing on the stability of the *secE-nusG* and ribosomal protein transcripts was studied. By S1 nuclease mapping, the decay of transcripts was monitored at two minute intervals in rifampicin-

treated *E. coli* cultures that either had a temperature-sensitive RNase E mutation or was deficient in RNase III activity. When the RNase E^{ts} strain was examined at the permissive temperature, the transcripts decayed normally with half-lives resembling those seen in the isogenic wild type parent. But when the strain was shifted to the restricted temperature, all the transcripts probed decayed more slowly, by a factor greater than 10-fold. In the absence of RNase III activity, only a slight stabilization of the transcripts was observed.

The results suggest that RNase E cleavage plays a significant role in the degradation of the *secE-nusG* and ribosomal protein transcripts either as the rate determining step initiating decay and/or as a secondary step that indirectly influences decay.

Furthermore, and perhaps most importantly, these observations can be used to demonstrate that the synthesis rates of these mRNAs are severely reduced when their degradation mediated by RNase E, and to a lesser extent, RNase III, is prevented. That is, the increase in the mRNA half-life is almost exactly compensated by a concomitant reduction in the mRNA synthesis rate such that the amount of mRNA remains virtually constant. How amount, half-life, and synthesis rates of mRNA are related is unknown.

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ABBREVIATIONS

A	adenosine
A_{460}	absorbance at 460 nm
ATP	adenosine triphosphate
bp	base pair
°C	degrees centigrade (Celcius)
C	cytosine
Ci	Curies
cpm	counts per minute
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	ethylene diamine tetraacetic acid
EtBr	ethidium bromide
G	guanosine
kb	kilobase
kDa	kilodalton
mRNA	messenger ribonucleic acid
NAD	nicotinamide adenine dinucleotide
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
PEG	polyethylene glycol
PNK	polynucleotide kinase
RNase	ribonuclease
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
S	Svedberg unit of sedimentation coefficient

SDS	sodium dodecyl sulphate
ss	single-stranded
T	thymidine
TAE	40 mM Tris-acetate, 1 mM EDTA
TBE	90 mM Tris-borate, 2 mM EDTA
TE	10 mM Tris-Cl (pH 7.5), 1 mM EDTA
Tris	tris-(hydroxymethyl)amino methane
tRNA	transfer ribonucleic acid
ts	temperature-sensitive
U	uridine
u	units
UTR	untranslated region
YT	8g/l Bacto tryptone, 5g/l Bacto yeast extract, 5g/l NaCl

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I.   INTRODUCTION

1.1   MESSENGER RNA DECAY

Messenger RNA decay is a naturally occurring biological process that is found in both eukaryotes (Atwater *et al.*, 1990) and eubacteria (Kennell, 1986). This process plays an essential role in determining levels of protein synthesis by limiting the concentration of the transcripts in the cell. Despite its importance in regulating gene expression, scientific effort has been mainly directed towards transcriptional and translational regulatory mechanisms rather than the mechanisms governing mRNA decay. As a result, this process is poorly understood.

The stability of messenger RNA varies significantly among species. Typically, in eukaryotic cultured cells, mRNA half-lives range from twenty five minutes in yeast to sixteen hours in humans (Kennell, 1986). Eubacteria have much shorter mRNA half-lives ranging from thirty seconds to three minutes for most transcripts (Brawerman, 1989). Short half-lives most likely reflects the ability of bacteria to rapidly alter their protein synthesis to adapt to fluctuating environments (Georgellis *et al.*, 1992). The longest-lived known eubacterial primary transcript, *ompA*, which encodes an outer membrane protein in *E. coli*, has a half-life of only 15 minutes (Nilsson *et al.*, 1984). The abundance of the ompA protein is a direct consequence of the strength of the promoter and the relatively long half-life of its transcript (von Gabain *et al.*, 1983).

One of the earliest and most striking properties of mRNA discovered was its inherent metabolic instability (Kennell, 1986). Unlike transfer RNA and ribosomal RNA which are stable for at least several generations, mRNA is extremely unstable and decays within one cell doubling time (King and Schlessinger, 1987).

When discussing mRNA decay, a distinction must be made between the functional and chemical decay of transcripts. The functional stability of a transcript is measured by the average time a transcript can actively direct the synthesis of structurally and functionally complete polypeptides. Experimentally, the functional stability of a transcript can be estimated from the ability of the mRNA to direct the synthesis of proteins after transcription initiation has been blocked with antibiotics such as rifampicin. Chemical decay is defined as the degradation of mRNA to oligonucleotides and mononucleotides. Chemical decay often follows functional inactivation, although functional and chemical decay are not always linked processes (Ono and Kuwano, 1979, Newbury *et al.*, 1987b).

1.2   MECHANISMS OF BACTERIAL MESSENGER RNA DECAY

Although the mechanistic details of transcript decay have not been elucidated, it is generally accepted that mRNA decay proceeds by a combination of endonucleolytic and exonucleolytic action (Belasco and Higgins, 1988). The stability of a transcript *in vivo* results from its susceptibility to attack by these enzymes, and is influenced by sequences and structures in the RNA as well as by its association with other molecules.

1.2.1   OVERVIEW OF THE DECAY PROCESS

For some transcripts, degradation occurs solely by the processive action of 3'-5' exonucleases, beginning at 3' ends generated by transcription termination (Mackie, 1989). This decay is very rapid but only in the absence of the 3' secondary structures characteristic of termination regions. These secondary structures act like barriers and impede the 3'-5' exonucleolytic activity. In most cases, the rate at which the exonucleases overcome the barrier is too slow and

another degradation pathway involving endonucleases is adopted. In the simplest case, an alternative pathway involves an initial endonucleolytic cleavage event that removes the secondary structure, allowing exonucleases to resume degradation from the newly formed 3' end. Another pathway involves an endonucleolytic cleavage initiated in the 5' region of the mRNA molecule which triggers subsequent endonucleolytic cleavage events along the transcript. The smaller fragments generated can then be degraded rapidly by 3'-5' exonucleases. A similar model can be envisioned for endonucleolytic events initiating decay in the middle of transcripts. Most messages appear to decay in a net 5'-3' direction, but a 5'-3' exonuclease has yet to be identified (Cannistraro and Kennell, 1985, Kennell, 1986, Belasco and Higgins, 1988).

1.2.2 EXONUCLEASE ACTIVITY

The involvement of two exonucleases in mRNA degradation in *E. coli* is well documented (Donovan and Kushner, 1986). Ribonuclease II (RNase II) and polynucleotide phosphorylase (PNPase) processively degrade single-stranded RNA in a 3'-5' direction. Although they differ in their degradation mechanisms, both enzymes require a 3' hydroxyl group at the end of the RNA molecule. RNase II irreversibly hydrolyzes RNA to yield nucleoside-5'-monophosphates, whereas PNPase reversibly phosphorolyzes RNA (adds phosphates resulting in lysis, analogous to adding water in hydrolysis) to yield nucleoside-5'-diphosphates (Littauer and Soreq, 1982).

PNPase is encoded by the *pnp* gene and has been isolated from both eubacterial (Soreq and Littauer, 1977) and eukaryotic sources (Godefroy-Colburn and Grunberg-Manago, 1972). PNPase is unique in that it can polymerize random sequences from nucleoside diphosphates processively without template

requirements (Sulewski *et al.*, 1989). RNase II is encoded by the *rnb* gene and has been purified from *E. coli* (Gupta *et al.*, 1977).

Neither exonuclease is essential for viability, however double mutants (*pnp⁻*, *rnbᵗˢ*) are not viable at the nonpermissive temperature (Donovan and Kushner, 1986). These enzymes do not seem to be functionally equivalent or perform complementary functions since RNase II and PNPase seem to have different physiological effects. Guarneros and Portier (1991) studied the rate of degradation of the *sib* secondary structure in *int* mRNA of bacteriophage λ, and found that RNase II is impeded longer and is more sensitive to secondary structure than PNPase. RNase II is stalled longer than PNPase by repetitive extragenic palindromic (REP) sequences (Higgins *et al.*, 1988, McLaren *et al.*, 1991), and RNase II does not degrade tRNA *in vitro* (Guarneros and Portier, 1991). PNPase plays a major role in the degradation of S20 mRNA which encodes an *E. coli* ribosomal protein (Mackie, 1989). It is unclear whether this mRNA is a specific target of PNPase, or whether PNPase simply degrades most transcripts more efficiently than RNase II.

Mutants in PNPase or RNase II do not exhibit a dramatic effect on the average chemical half-life of pulse-labeled RNA (Donovan and Kushner, 1986). This suggests that these enzymes do not carry out the rate limiting step of the degradation of most mRNA molecules but are instead involved in the terminal steps of decay.

1.2.3 ENDONUCLEASE ACTIVITY

In *E. coli*, two endonucleases, RNase E and RNase III, have been implicated as participants in the decay of some transcripts. These enzymes were initially characterized for their processing function in cleaving the nascent rRNA primary transcript into precursor molecules (Figure 1). RNase E is required for
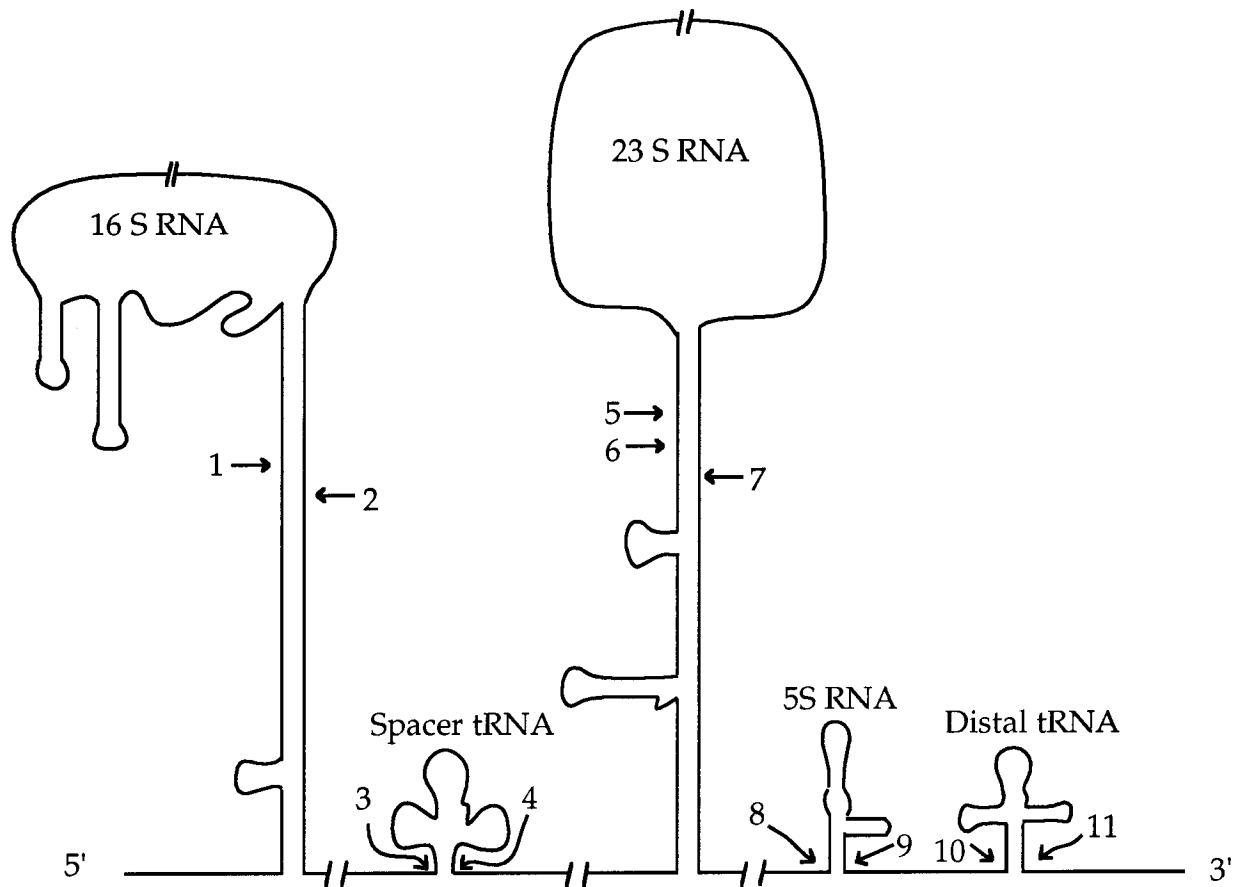
Figure 1: Schematic of the structure of an *E. coli rrn* primary transcript showing major processing sites for 16S, 23S, and 5S RNAs

The numbers indicate the positions of primary processing sites by RNase III and RNase E. Processing by RNase III releases precursor 16S RNA at sites 1 and 2, and precursor 23S RNA at sites 5, 6, and 7. RNase E cleaves a 9S precursor at sites 8 and 9, to form a 5S precursor molecule. The precursors are later matured into functional 16S, 23S, and 5S molecules. RNase P cleavages are indicated (3, 10) as well as RNase D cleavage sites 4 and 11. Taken from Srivastava and Schlessinger, (1990).

cleaving 9S rRNA to produce p5, a precursor which later matures to 5S rRNA

(Ghora and Apirion, 1978, Apirion, 1978, Apirion and Lassar, 1978, Misra and

Apirion, 1979, Roy *et al.*, 1983). A temperature-sensitive RNase E mutant fails to

produce 5S rRNA but instead accumulates the 9S precursor at the nonpermissive

temperature (Ghora and Apirion, 1979). Other natural substrates of RNase E

include RNAI, a noncoding stable RNA involved in controlling the copy

number of ColE1 plasmids (Tomcsanyi and Apirion, 1985), and bacteriophage T4

gene 32 and gene 59 mRNAs encoding the single-stranded DNA-binding

proteins essential for replication, recombination and repair of T4 DNA (Mudd *et*

al., 1988, Carpousis *et al.*, 1989). RNase E-dependent processing is also observed

in the mRNA of the *pap* operon, encoding proteins required for expression of P-

fimbrial adhesions in *E. coli* (Nilsson and Uhlin, 1991), the *rpsO* mRNA,

encoding the ribosomal protein S15 (Regnier and Hajnsdorf, 1991), and *unc*

mRNA, encoding eight subunits of F_1F_0-ATPase in *E. coli* which couples

translocation of protons across the membrane to ATP synthesis in oxidative

phosphorylation (Patel and Dunn, 1992).

The first endoribonuclease identified as having a general role in the

degradation of mRNA in *E. coli* was RNase E. A temperature-sensitive

mutation in the altered mRNA stability (*ams*) gene was first described for its

effect on the chemical half-life of total pulse-labeled RNA (Kuwano *et al.*, 1977,

Ono and Kuwano, 1979). Since then, the *rne* gene encoding RNase E (Chauhan

and Apirion, 1991) has been identified to be the same genetic locus as the *ams*

gene by studies using a temperature-sensitive mutant, *rne*[ts] (Taraseviciene *et al.*,

1991). Both the *ams*[ts] and *rne*[ts] mutations have the same affect on bulk mRNA

decay and rRNA processing (Melefors and von Gabain, 1991, Babitzke and

Kushner, 1991), and both mutations are complemented by a recombinant

plasmid carrying the *ams* gene. Functional RNase E may consist of both protein

and a tightly bound RNA component (Chauhan *et al.*, 1991), although the nature of the RNA is not clear. The only other precedent for an RNA moiety in an RNA processing enzyme is found in RNase P, which processes tRNA (Stark *et al.*, 1978). The gene for RNase E has been cloned (Claverie-Martin *et al.*, 1989) and the protein has been partially purified and characterized (Misra and Apirion, 1979, Roy *et al.*, 1983, Roy and Apirion, 1983). The cellular importance of RNase E is indicated by the findings that the only *rne* mutations isolated are conditionally lethal, and that multicopies of the *rne* gene results in toxicity to the cell (Claverie-Martin *et al.*, 1989). The specificity of this endonuclease is not clearly understood although mutational analyses and sequence comparisons between known substrates of RNase E have resulted in a loosely defined consensus sequence (G/A)AUU(A/U) followed by a downstream stem loop structure (Ehretsmann *et al.*, 1992).

The other endonuclease involved in decay is RNase III, encoded by the *rnc* gene. RNase III is a homodimer of two 25kDa subunits (Dunn, 1976), and it has been well characterized for mediating the separation of 16S and 23S precursors from the primary transcript (Figure 1, p5). In an RNase III mutant, 23S precursors are incompletely matured, yet still function normally as a longer pre-rRNA species, and 16S precursors are matured normally at the same rate by other primary and secondary processing enzymes (Gegenheimer *et al.*, 1977, Srivastava and Schlessinger, 1989). RNase III recognizes a sequence-independent secondary structure and cleaves in the single-stranded or double-stranded regions on one or both sides of the stem (Chelladurai *et al.*, 1991). It has been suggested that the length of the continuous pairing of the secondary structure has some role in determining specificity (Krinke and Wulff, 1990). RNase III is not essential for *E. coli* viability (Takiff *et al.*, 1989), although one mutant allele displays markedly pleiotropic effects on plasmid replication, phage growth, and quantity of *E. coli*

proteins synthesized (King *et al.*, 1986). The extent of RNase III involvement in general mRNA decay has not been resolved, however RNase III cleavage in the 5' noncoding region has been found to be the rate limiting step in the decay of a number of transcripts such as the *rpsO-pnp* (Portier *et al.*, 1987), *rnc-era* (Bardwell *et al*, 1989), *metY-nusA-infB* operons (Regnier and Grunberg-Manago, 1990) and the *int* gene of phage λ (Guarneros *et al.*, 1982, Schmeissner *et al.*, 1984). As well, RNase III processing increases translational activity of mRNA by disrupting secondary and tertiary structures that inhibit ribosome binding of some phage T7 mRNAs (Dunn and Studier, 1975) and of the bacteriophage λ *cIII* gene (Altuvia *et al.*, 1987).

1.2.4   FACTORS INFLUENCING DEGRADATION RATES

The lifetime of most transcripts is determined by their susceptibility or resistance to degradation. Features such as secondary structures at the 3' and 5' ends and in intergenic regions, stem loop binding proteins, and ribosomes seem to have an important role in determining sensitivity to nucleolytic attack. Depending on which factors are present, the lifetime of the transcript will either be enhanced or reduced.

1. Secondary Structures At The 3' End Of Transcripts And In Intergenic Regions

Rho-independent transcription terminators and secondary structures formed by repetitive extragenic palindromic (REP) sequences at the 3' end of transcripts form effective barriers against 3'-5' exonucleolytic degradation. *In vitro*, the *trp t* terminator of the *trp* operon blocks RNase II (Mott *et al.*, 1985), the *tI* terminator of the *int* gene in bacteriophage λ blocks PNPase (Guarneros and Portier, 1991), and studies with strains mutant in RNase II and PNPase activity

show that terminators and stem loop structures are important for blocking the exonucleolytic activity in S20 and *glyA* mRNA (Mackie, 1989, 1992).

REP sequences have been shown to protect mRNA from PNPase and RNase II degradation (Higgins *et al.*, 1988). These palindromic sequences, which are present in 500 to 1000 copies on the *E. coli* chromosome, have a high potential to form RNA secondary structures (Higgins *et al.*, 1982, 1988, Gilson *et al.*, 1984). Since REP sequences are present in approximately 25% of all transcription units, this determinant likely confers stability on a number of transcripts by protecting the transcripts from 3' exonucleolytic degradation. It has been shown that these REP sequences can confer increased stability on upstream *malE* mRNA as well as on heterologous transcripts *in vivo* (Newbury *et al.*, 1987a,b, McLaren *et al.*, 1991).

In polycistronic operons, both intergenic stem loops and stabilizing structures formed by REP sequences allow selective degradation of downstream segments of mRNA and account for differential gene expression. This has been observed in *E. coli* operons *malEFG* (Newbury *et al.*, 1987a), *lac* (Cannistraro *et al.*, 1986), *ars* (Owolabi and Rosen, 1990), *pap* (Baga *et al.*, 1988, Nilsson and Uhlin, 1991), and *atp* (McCarthy *et al.*, 1991, Ziemke and McCarthy, 1992), the *Salmonella typhimurium his* transport operon (Newbury *et al.*, 1987b), and the *puf* operon of *Rhodobacter capsulatus* (Klug *et al.*, 1987, Chen *et al.*, 1988).

2. The 5' Non-coding Region

The exact mechanism by which the 5' noncoding region influences mRNA decay is not understood. Many mRNAs appear to be degraded via a pathway in which the 5' noncoding region performs a protective function against the agents of decay. This model is supported by the finding that the 5' untranslated region of *ompA* mRNA (Melefors and von Gabain, 1988, Emory *et*

al., 1992), and bacteriophage T4 gene 32 mRNA (Mudd *et al.*, 1988) can confer stability on labile transcripts. More specifically, using *ompA* mRNA (Chen *et al.*, 1991, Emory *et al.*, 1992) and RNAI (Bouvet and Belasco, 1992), it was found that a sequence-independent stem loop structure, at or within two unpaired nucleotides of the 5' terminus, is responsible for preventing degradation initiated internally by a 5' end-dependent endonuclease. In the case of RNAI, it is thought that RNase E preferentially binds to the single-stranded nucleotides at the 5' end, or to another protein already bound there, and cleaves the mRNA just downstream at the RNase E recognition site. This pathway could explain how degradation of some messages is controlled by the 5' end without the need for invoking a 5' exonuclease. As well, RNAI is not translated in *E. coli* (Bouvet and Belasco, 1992) so degradation, in this case, is not a consequence of translating ribosome activity (discussed below).

3. Ribosomes

The evidence for the role of ribosomes in transcript stability is rather controversial. One model suggests that an initial endonucleolytic cleavage in the translation initiation region disrupts ribosome loading leading to ribosome depleted stretches that fail to protect the mRNA from further endonucleolytic attack or processive degradation from the 5' end. In the *E. coli atpE* gene, disruption of the translation initiation region leads to enhanced decay rates for the upstream region, presumably due to 3'-5' exonucleases, and the downstream region also exhibits an increased rate of decay (Gross, 1991). As well, 5' regions from transcripts with low translational efficiencies such as the *tetR* gene can destabilize hybridized transcripts (Baumeister *et al.*, 1991).

A related model suggests that the spacing between ribosomes exposes the mRNA to endonucleolytic attack since a complete absence of ribosomes seems to

cause a shorter mRNA half-life (Nilsson *et al.*, 1988), and a block of translation initiation by kasugamycin leads to an increase in chemical decay of *lac* mRNA (Schneider *et al.*, 1978). However, removal of almost all of the 5' noncoding region upstream of the ribosomal binding site in *ompA* transcripts results in a marked instability of the mRNA, but only marginally reduces the frequency of translation initiation (Emory and Belasco, 1990) suggesting that the mechanism of stabilization of this mRNA is not related to the spacing between ribosomes. As well, no difference in the stabilities of translated and untranslated segments of the *ompA* and *bla* mRNAs have been detected (von Gabain *et al.*, 1983). It seems that ribosome spacing may influence the stability of some transcripts but it is important not to generalize about the vulnerability of all untranslated segments of transcripts.

4. Stem Loop Binding Proteins

In spinach chloroplasts, proteins have been identified that specifically bind to the stem loop structures located at the 3' end of transcripts and therefore block exonucleolytic activity (Stern *et al.*, 1989). Although there are no prokaryotic examples of stem loop binding proteins, McLaren *et al.* (1991) suggest that such a factor may exist. Their data shows that stem loop structures are sufficient to block exonucleolytic activities, but that the structure alone is insufficient to account for the length of time that stability is conferred *in vivo*. They suggest that a stem loop binding protein provides a more long term blockage to exonucleolytic attack and that the transcripts are then degraded by a pathway involving endonucleases.

Factors that affect the susceptibility of mRNA to decay do not necessarily function independently. Klug and Cohen (1990) have determined that a combination of multiple hairpin structures and rate limiting endonucleolytic

cleavage sites together determine differential degradation rates of individual messages within the polycistronic *puf* operon. With further investigation into the factors affecting decay, concerted influence may prove to be the norm rather than the exception.

1.3   EUKARYOTIC MESSENGER RNA DECAY

Messenger RNA decay is also prevalent in eukaryotic systems but has a much more complex regulatory context than in bacterial systems. Stability of transcripts can be influenced in response to developmental programmes or to external stimuli such as hormones, lymphokines, oncogenes, and transcriptional activators (Atwater *et al.*, 1990, Carter and Malter, 1991, Hentze, 1991). Most eukaryotic studies have focussed on identifying stability determinants such as the 5' cap, the poly(A) tail, the poly(A) tail binding protein, 3' UTR A-U rich sequences, 3' UTR binding proteins, and other structural elements (Greenberg *et al.*, 1990, Brewer, 1991, Weiss *et al.*, 1991, Bernstein *et al.*, 1992, Lieberman, *et al.*, 1992). As in bacterial systems, the degradation pathway invokes endonucleases and exonucleases, although no nucleases have been identified that play a degradative role in eukaryotes.

1.4   GENOMIC ORGANIZATION OF THE RIF REGION

The *secE-nusG* and the *rplKAJL-rpoBC* operons are located adjacent to each other in the rif region near 90 minutes on the *E. coli* chromosome (Figure 2, p 25). The *secE* and *nusG* genes are separated by a single nucleotide and are cotranscribed with transcription initiation occurring at the P_{EG} promoter. Transcription is efficiently terminated at a rho-independent terminator located in the *nusG*-L11 intergenic space downstream of the P_{L11} promoter (Downing *et al.*, 1990). The *secE* gene codes for a 127 amino acid long integral membrane

protein that is a component of the translocation apparatus involved in exporting proteins across the cytoplasmic membrane (Riggs *et al.*, 1988, Schatz *et al.*, 1989, Stader *et al.*, 1989). The *secE* gene product is essential for viability, as indicated by the isolation of a cold-sensitive mutant (Schatz *et al.*, 1989). The *nusG* gene encodes a 181 amino acid long polypeptide that is involved in the λ N-mediated transcription antitermination system (Sullivan *et al.*, 1992). Downing *et al.* (1990) have shown that *nusG* is also essential for viability.

The *rplKAJL-rpoBC* gene cluster located immediately downstream of the *secE-nusG* operon has been well characterized in terms of its genomic organization, transcription patterns, and regulation (Downing and Dennis, 1987). The major transcript is a tetracistronic message encoding four 50S ribosomal subunit proteins; L11 (*rplK*), L1 (*rplA*), L10 (*rplJ*) and L12 (*rplL*), initiated at the P_{L11} promoter. Approximately 80% of the transcripts are attenuated in the L12-β intergenic region and the remaining 20% read through the attenuator sequence and transcribe the downstream *rpoB* and *rpoC* genes, encoding the large β and β' subunits of RNA polymerase. Transcription is also initiated from the P_{L10} promoter, which cotranscribes the *rplJ* (L10) and *rplL* (L12) genes.

1.5   PURPOSE OF THIS PROJECT

Nuclease S1 protection assays and primer extension analyses in the rif region identified several 5' ends of transcripts that were not initiated at any promoters (Downing and Dennis, 1987, Downing *et al.*, 1990). Preliminary experiments suggested that these 5' ends may be a result of RNase E or RNase III processing. The purpose of this work is to confirm the identity of these putative processing sites and determine what role, if any, RNase E and RNase III endonuclease processing events have in mRNA decay in the rif region on the *E. coli* chromosome.

II. MATERIALS AND METHODS

2.1 BACTERIAL STRAINS AND PLASMID CONSTRUCTIONS

The bacterial strains and plasmid constructions that were used are described in Table 1. The *ams1* allele in strains SK5665 and SK5671 encodes a thermolabile protein, and the *pnp7* allele is a nonsense mutation (Arraiano *et al.*, 1988). The wild type strain, MG1693, is the isogenic parent of SK5665 (*ams^{ts}*) and differs from N2076 (*rnc^+*), the isogenic wild type parent of N2077 (*rnc^-*) with respect to its nutritional requirements.

2.2 MEDIA AND CULTURE CONDITIONS

Bacteria were grown in YT media (8 g/l Bacto tryptone, 5 g/l Bacto yeast extract, 5 g/l NaCl) with 50 µg/ml thymine, or in M9 minimal salts (Miller, 1972) supplemented with 0.2% glucose, 0.5 µg/ml thiamine and 50 µg/ml amino acids or 1 µg/ml NAD when required. The cultures were shaken in a rotary bath or air shaker at 37°C, or at 30°C for the temperature-sensitive mutants, and growth was monitored by measuring the absorbance at 460 nm. When required, ampicillin (100 µg/ml) or tetracycline (50 µg/ml) was added to media or plates.

2.3 GENERAL MOLECULAR BIOLOGY TECHNIQUES

The general molecular biology techniques used can be found in Sambrook *et al.* (1989) unless otherwise specified.

Table 1: Bacterial strains and plasmid constructions

Strain	Description
C600 (PD26) Copenhagen	*thr leu trp thi recA*
DH5α	F⁻ *recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* λ⁻
RB404	*dcm dam*
MG1693 (PD952)	*thyA715* (from S. Kushner)
SK5665 (PD953)	*ams1*^{ts} *thyA715* (from S. Kushner)
SK5671 (PD954)	*ams1*^{ts} *pnp7 thyA715* (from S. Kushner)
SK5691 (PD955)	*pnp7 thyA715* (from S. Kushner)
N2076 (PD672)	F⁻ *thi argH1 nad84 lacY1 gal6 nalA1* λ^r*xyl7 ara13 mtl2 str9 tonA2* (from D. Apirion)
N2077 (PD673)	F⁻ *thi argH1 nad84 lacY1 gal6 nalA1* λ^r*xyl7 ara13 mtl2 str9 tonA2 rnc105* (from D. Apirion)

Plasmid	Description
pBRU (PD828)	*Sma*I-*Eco*RI 2.1kb fragment containing the 3' end of the *tufB* gene, the entire *secE* and *nusG* genes and the 5' end of the *rplK* (L11) gene, cloned into the *Eco*RI and the blunt-ended *Cla*I sites of pBR322 (from W. Downing)
pUB1108 (PD1058)	*Hind*III-*Hpa*I 1.1kb fragment containing the 3' end of the *tufB* gene, and the 5' end of the *secE* gene, cloned into the *Hind*III and *Sma*I sites of pGEM-7Zf(+)

pUG570 (PD1059)

*Pst*I 570bp fragment containing the 3' end of the *secE* gene and the 5' end of the *nusG* gene, cloned into the *Pst*I site of pGEM-3Zf(+)

p1344 (PD255)

*Pst*I 6.1kb fragment from λdrif^{d}18, containing all the ribosomal protein genes as well as the 5' end of the *rpoB* (β) gene, cloned into pBR322 (from J.D. Friesen)

pUL11 (PD1060)

*Eco*RI-*Bgl*II 617bp fragment containing the 3' end of the *rplK* (L11) gene and the 5' end of the *rplA* (L1) gene, cloned into the *Eco*RI and*Bam*HI sites of pGEM-3Zf(+)

pUL10 (PD1061)

*Pst*I-*Hind*III 359bp fragment containing the inner majority of the *rplJ* (L10) gene, cloned into the *Pst*I and *Hind*III sites of pGEM-3Zf(+)

pUB (PD1062)

*Pst*I-*Acc*I 348bp fragment containing a small region of the *rpoB* (β) gene, cloned into the *Pst*I and *Acc*I sites of pGEM-3Zf(+)

2.3.1 PLASMID DNA PREPARATION

Small and large scale plasmid DNA was prepared by the alkaline lysis procedure (p1.25, 1.38, Sambrook *et al.*, 1989) with the following modifications in the large scale procedure. Plasmids were amplified overnight in the presence of chloramphenicol (170 µg/ml). Lysozyme was omitted, and the supernatant was not filtered through layers of cheesecloth. Instead, after the isopropanol precipitation, the pellet was resuspended in 2 ml TE and phenol:chloroform extracted. The ethanol precipitated DNA was resuspended in 6 ml of water and 10 M LiCl (final concentration of 3 M LiCl) to precipitate high molecular weight RNA. After centrifugation, ethanol was added to the supernatant to precipitate the DNA. The final DNA pellet was dissolved in 0.97x CsCl (made by dissolving 97 g CsCl in 100 ml water) and was purified by equilibrium centrifugation in a CsCl-ethidium bromide (EtBr) gradient as described on p1.42, Sambrook *et al.* (1989). The density gradients were centrifuged at 100 000 rpm for 3 hours in a TLV-100 rotor in the Beckman TL-100 tabletop ultracentrifuge. The EtBr was removed from the purified DNA by extraction with organic solvents and the CsCl was removed by diluting the DNA solution with 3 volumes water and precipitating the DNA with 2 volumes of 95% ethanol.

2.3.2 RESTRICTION ENDONUCLEASE DIGESTION OF DNA

Restriction endonuclease digestions were performed according to the instructions of the suppliers.

2.3.3 DEPHOSPHORYLATION OF IDENTICAL COHESIVE TERMINI OF VECTOR DNA

Identical cohesive termini of vector DNA were dephosphorylated by shrimp alkaline phosphatase (3u/µg DNA) (United States Biochemical). The

enzyme was incubated in SAP buffer (20 mM Tris-Cl, pH 8.0, 10 mM MgCl$_2$) with the DNA for 1 hour at 37°C to prevent religation. Shrimp alkaline phosphatase was heat inactivated at 65°C for 15 minutes.

2.3.4 GEL ELECTROPHORESIS

Agarose gels (0.7% or 2%) with 0.5 µg/ml EtBr were run in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, 0.5 µg/ml EtBr) at 125 V.

Preparative (3 mm thick) 5% polyacrylamide gels were run in TBE buffer (90 mM Tris-borate, 2 mM EDTA) at 250 V, and stained in 0.5 µg/ml EtBr after electrophoresis.

2.3.5 PREPARATION OF RESTRICTION DIGESTED DNA FRAGMENTS

DNA fragments electrophoresed on agarose gels were excised for cloning and purified by the Sephaglas BandPrep Kit (Pharmacia).

DNA fragments electrophoresed on polyacrylamide gels were excised for S1 mapping, placed in dialysis tubing and electroeluted for 1-2 hours at 150 V in TAE buffer. DNA contained in the eluate was purified by phenol:chloroform extraction and recovered by ethanol precipitation.

2.3.6 LIGATIONS

Excess insert DNA was ligated to 25 ng vector DNA (4:1 mass ratio) using T4 DNA ligase (Pharmacia). The ligase buffer included 5% PEG for both blunt-end and sticky-end ligations which allowed incubation at room temperature for 1 hour. The total ligation volume was 20 µl, of which half was used per transformation.

2.3.7 TRANSFORMATIONS

Fresh competent *E. coli* cells were prepared using calcium chloride (p1.82, Sambrook *et al.*, 1989). Half of the ligation mix was added to an aliquot of competent cells and incubated on ice for 20-30 minutes. The cells were heat shocked at 42°C for 90 seconds, plated on selective media, and incubated at 37°C for 12-16 hours.

2.3.8 5' END-LABELING OF DNA FRAGMENTS

DNA fragments (150 ng) with protruding 5' termini were dephosphorylated as in Section 2.3.3 using shrimp alkaline phosphatase, and end-labeled using bacteriophage T4 polynucleotide kinase (T4 PNK) (Pharmacia) and [γ-^{32}P]ATP (sp. act. 3000 Ci/mmol, 10 μCi/μl) (p10.60 Sambrook *et al.*, 1989). Bacteriophage T4 PNK was heat inactivated at 65°C. Excess radiolabel was removed by ethanol precipitation and the radioactive DNA fragments were measured by Cherenkov counting.

2.3.9 3' END-LABELING OF DNA FRAGMENTS

DNA fragments with recessed 3' termini were end-labeled using the Klenow fragment of *E. coli* DNA polymerase I and appropriate [α-^{32}P]dNTP according to p10.52, Sambrook *et al.* (1989). The molecular weight size standard, pBR322 digested with *Msp*I, was also 3' end-labeled by the same method.

2.4 TOTAL CELLULAR RNA PREPARATION

2.4.1 GENERAL METHOD

E. coli strains were grown in YT media or M9 minimal salts, as indicated in Section 2.2 (A_{460}=0.3-0.4). The cellular activities of 25 ml samples were

arrested with the addition of 200 µl of 1 M NaN_3 and samples were centrifuged at 9000 rpm for 5 minutes. The pellet was resuspended in 1 ml solution C (40 mM NH_4Cl, 40 mM Na_2HPO_4, 20 mM KH_2PO_4, 50 mM NaCl) and 10 mM NaN_3, added to 1 ml boiling SDS lysis mix (100 mM NaCl, 10 mM EDTA, 0.5% SDS) and boiled for 30 seconds. The lysate was immediately extracted 3 times with an equal volume of phenol and once with chloroform, and the RNA was precipitated with ethanol and 0.25 M NaCl. The RNA pellet was resuspended in 0.87 ml TE and 0.43 g CsCl and layered onto a 0.33 ml cushion of 5.7 M CsCl in polycarbonate tubes. The RNA was pelleted through the CsCl gradient by ultracentrifugation at 100 000 rpm for 3 hours in a TLA100.2 rotor. The pellet was resuspended in DEPC-treated water, and the RNA was precipitated with ethanol. RNA concentrations were measured by absorbance readings at 260 nm where 1OD=40 µg/ml. RNA preparations (5 µg) were analyzed on a 1% agarose gel as described in Section 2.3.4.

2.4.2 RNA PREPARATIONS OF TEMPERATURE-SENSITIVE MUTANTS

Total RNA preparations of the temperature-sensitive mutant strains were done as described in Section 2.4.1 with the exception of the growth conditions. Strains MG1693, SK5665, SK5671, and SK5691 (Table 1) were initially grown at the permissive temperature of 30°C. MG1693 is a wild type strain; SK5665 has a temperature-sensitive mutation in the *ams* (altered message stability) gene which is lethal at the restrictive temperature of 44°C; SK5671 is a double mutant carrying the temperature-sensitive *ams* mutation, as well as a mutation in the *pnp* (polynucleotide phosphorylase) gene. PNPase is an exonuclease involved in the 3'-5' decay of some transcripts. SK5691 has a single mutation in the *pnp* gene.

All four strains were grown at 30°C and shifted at appropriate times during early log phase to allow incubation for 1 hour at the restrictive temperature of 44°C. RNA was isolated at the permissive and restrictive temperatures according to Section 2.4.1.

2.5 S1 NUCLEASE MAPPING

The 5' ends of *in vivo* transcripts were analyzed by S1 nuclease mapping. Total cellular RNA (20 µg) was hybridized to 10^5 cpm of 3' or 5' radioactively labeled denatured DNA fragments in 80% deionized formamide and 5x hybridization buffer (200 mM PIPES, pH 6.8, 2 M NaCl, 5 mM EDTA) for 3 hours at 52°C. Single-stranded nucleic acids were digested with S1 nuclease (400u/ml) at 37°C for 30 minutes, and sizes of the protected RNA-DNA hybrids were determined on an 8% polyacrylamide DNA sequencing gel run at 32 W, with 3' end-labeled pBR322-*Msp*I as a molecular length standard.

2.6 MESSENGER RNA STABILITY

To measure the stability of transcripts at permissive and restrictive temperatures, cells were grown according to Section 2.4.1 for strains C600, N2076 and N2077, and according to Section 2.4.2 for the temperature-sensitive strains. When the cultures reached $A_{460}=0.3-0.4$, a time zero sample was taken simultaneously with the addition of rifampicin (final concentration 200 µg/ml) to the remaining culture. Successive 25 ml samples were taken at 2 minute intervals over a period of 14 minutes. Total RNA was isolated from these samples and analyzed for levels of *secE*, *nusG* , L11, L10, and β transcripts by S1 nuclease mapping as described in Section 2.5. The resultant autoradiograms were scanned on a Bio-Rad model 620 scanning densitometer to construct mRNA decay curves.

2.7   RELATIVE AMOUNT OF mRNA SEQUENCES

The relative amounts of *secE*, *nusG*, L11, L10, and β transcripts were analyzed by S1 mapping (Section 2.5) before rifampicin was added to the cultures. A constant amount of RNA (either 10 or 20 µg) was hybridized to the radiolabeled DNA and treated with S1 nuclease. The protected fragments were visualized by autoradiography and the relative intensities of the bands representing a given mRNA sequence hybridizing to a specific DNA probe were determined by densitometry. These relative amount measurements are not comparable between different mRNA sequences.

2.8   RELATIVE SYNTHESIS RATES

The relative rates of synthesis of mRNA sequences were calculated from the relative amounts of mRNA and the mRNA half-lives using the first order rate equation:

$$dx/dt = X \ln2/\tau_1 + X \ln2/\tau_2$$

where: dx/dt is the relative instantaneous synthesis rate of an mRNA sequence
  X is the amount of the mRNA sequence
  τ_1 is the half-life of the mRNA sequence
  τ_2 is the mass doubling time of the culture

Because the mass doubling time is long, its contribution to the instantaneous synthesis rate of mRNA is small and therefore, can be ignored. For example, if the average half-life of all mRNA is 2 minutes and the mass doubling time is 40 minutes, 95% of the mRNA synthesis rate represents replacement of the decaying transcripts whereas only 5% represents growth of the culture. For shorter half-lives, and larger doubling times, the growth component is even smaller. The equation that is used in this work is:

$$dx/dt = X \ln2/\tau_1$$

III. RESULTS

The mutant and wild type *E. coli* strains were tested for their growth characteristics. The *ams1*^{ts} mutant (SK5665), its isogenic parent (MG1693), the *pnp7* mutant (SK5691), and the *ams1*^{ts} *pnp7* double mutant (SK5671) were all able to grow at 30°C. When the strains were grown in liquid media at the same temperature, doubling times of 35-40 minutes were observed with the exception of SK5671, which exhibited slower growth with a doubling time of 60 minutes (Table 2). When the strains were grown on plates directly at the nonpermissive temperature of 44°C, the temperature-sensitive strains SK5665 and SK5671 failed to grow. In liquid media, when these temperature-sensitive strains were shifted to the nonpermissive temperature, the growth rate initially increased and than gradually decreased over a period of one generation as the cells. This result confirmed the expected genotypes of the strains and was comparable to the results obtained by Arraiano *et al.* (1988). As well, the *rnc* mutant (N2076) and its isogenic parent (N2077) grew at 37°C with doubling times of 60 and 35 minutes respectively, in liquid media.

The genomic organization of the rif region of the *E. coli* chromosome is illustrated in Figure 2. Previously, S1 nuclease mapping and primer extension analysis in this region revealed several 5' transcript ends (denoted by scissors in Figure 2) that were not initiated at promoters, but were a result of processing (Downing and Dennis, 1987, Downing *et al.*, 1990). To determine whether these 5' ends were generated by either RNase E or RNase III cleavage, transcripts were analyzed in the presence and the absence of RNase E and RNase III activity.

strain	growth medium	temperature (°C)	doubling time (min)
MG1693 (ams⁺pnp⁺)	YT	30 44	35 25
SK5665 (ams1)	YT	30	40
SK5671 (ams1 pnp7)	YT	30	60
SK5691 (pnp7)	YT	30	35
N2076 (rnc+)	M9 + glucose	37	35
N2077 (rnc-)	M9 + glucose	37	60

Table 2: Growth conditions for the various *E. coli* strains

The mutant and wild type *E. coli* strains were grown in either YT (yeast tryptone) or M9 minimal media at their respective temperatures. Growth rates were measured by monitoring the cell density of exponentially growing cultures at 460 nm and are expressed as doubling times. The growth rate of MG1693 shifted to the restricted temperature of 44°C is indicated.
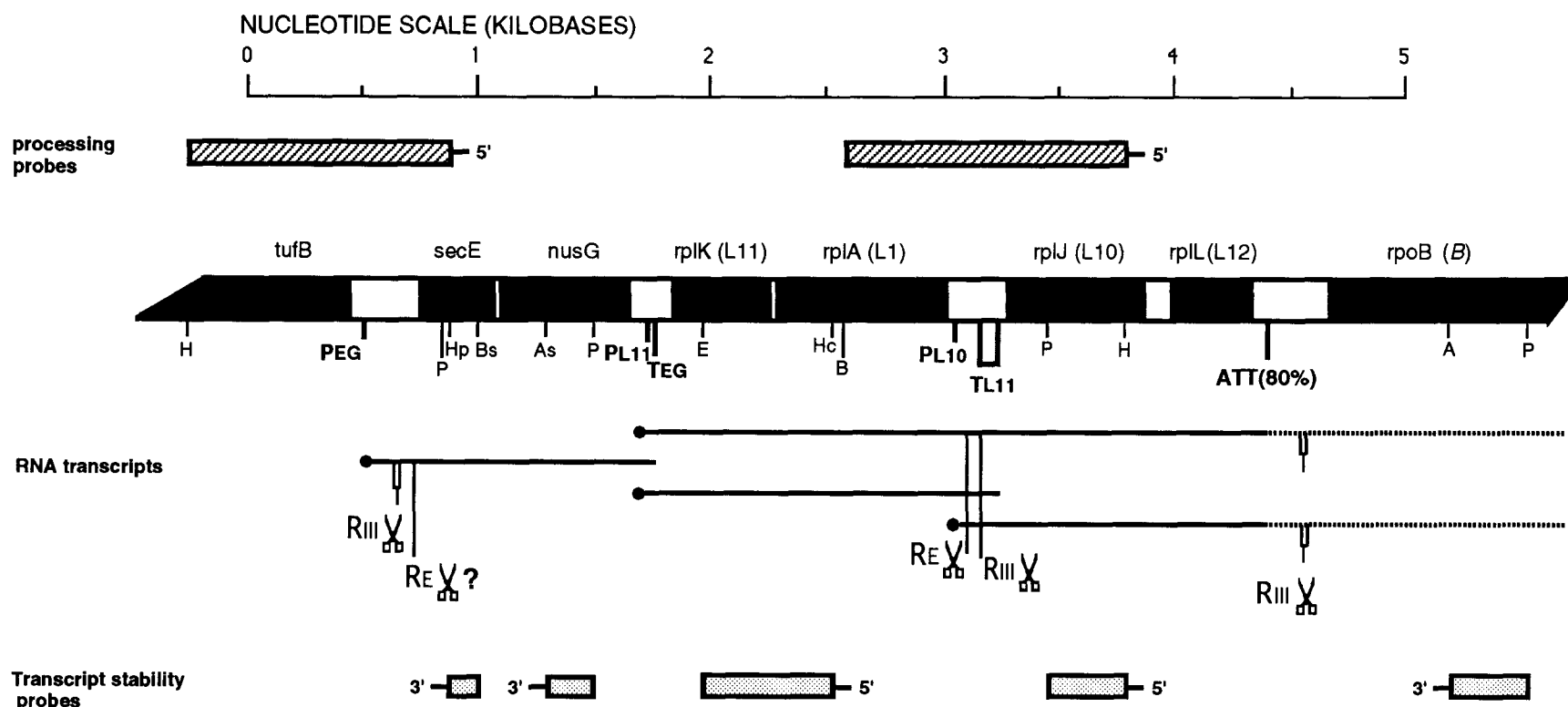
Figure 2: The genomic organization of the rif region on the *Escherichia coli* chromosome

The positions of the genes in the *secE-nusG* and ribosomal protein operons are denoted by black-filled rectangles. Transcripts initiated at promoters (PEG, PL11, and PL10) and terminated at terminators (TEG, TL11) are shown. The dashed lines indicate the 20% of transcripts that read through the attenuator (ATT). The putative RNase E and RNase III processing sites being examined are denoted by scissors. The 5' end-labeled probes used in S1 mapping to confirm the presence of the RNase E and RNase III sites are indicated above the map. The end-labeled probes shown below the map, were used to detect transcript stability. Relevant restriction endonuclease sites are indicated as *Hind*III (H), *Pst*I (P), *Hpa*I (Hp), *Bst*EII (Bs), *Asp*718 (As), *Eco*RI (E), *Hinc*II (Hc), *Bgl*II (B), *Acc*I (A).

A *Hind*III-*Hpa*I 1.1kb DNA probe spanning the *secE-nusG* leader and a *Bgl*II-*Hind*III 1.2kb DNA probe spanning the L1-L10 intergenic space shown in Figure 2, were used to detect the 5' ends of processed transcripts by S1 nuclease protection assay.

In Figure 3, transcripts initiated at the P_{EG} promoter resulted in a protected fragment of 352 nucleotides in length. Nucleotide lengths of the fragments were previously determined by primer extension analysis (Downing and Dennis, 1987, Downing et al., 1990). The next two largest fragments (315 and 282 nucleotides long) appear to be a result of RNase III cleavage since the intensity of these bands are reduced in an RNase III deficient strain (Figure 3, lanes 5 and 6) and the intensity of the full length fragment (352 nucleotide long band) increases accordingly. The remainder of the bands representing minor transcripts were detected in all the strains including C600, a well characterized wild type strain (data not shown), which contrasted with work by Downing et al. (1990) who previously detected a relatively intense band running at 195 nucleotides in length under identical experimental conditions.

To ensure that unknown experimental conditions did not somehow affect the processing at this site resulting in a weaker intensity band, several studies were done using C600 RNA for direct comparison to the work by Downing et al. (1990). However, modifying the inoculation dilution factor, duration of growth, type of media, DNA-RNA hybridization temperature during the S1 mapping procedure, and amounts of RNA, did not have any effect on the intensity of the band (data not shown).

Downing et al. (1990) suggested that this 195 nucleotide long fragment resulted from RNase E processing based on loose sequence similarity to other known RNase E cleavage sites. Using *E. coli* strains with a temperature-sensitive mutation in RNase E, (Figure 3, lanes 2 and 3), cleavage by RNase E at the
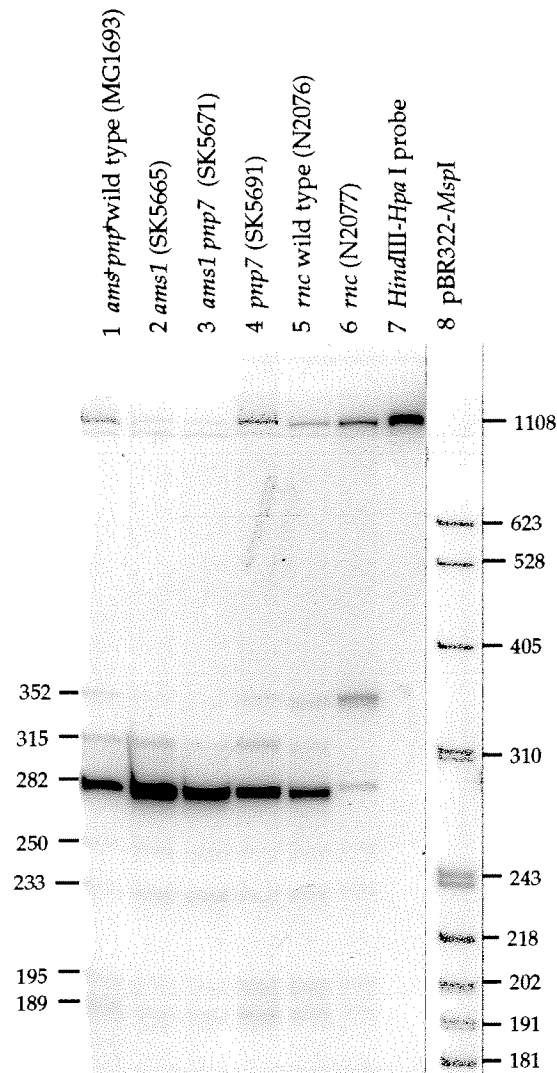
Figure 3: S1 nuclease protection analysis of *secE-nusG* transcripts

The 5' ends of transcripts in the *secE-nusG* leader were analyzed by S1 nuclease protection studies using a 5' end-labeled 1.1kb *Hind*III-*Hpa*I DNA fragment. Lane designations are: lane 1, MG1693 RNA, isogenic wild type parent (RNase E$^+$) ; lane 2, SK5665 RNA (RNase E^{ts}) at 44°C; lane 3, SK5671 RNA (RNase E^{ts}, PNPase$^-$) at 44°C; lane 4, SK5691 RNA (PNPase$^-$); lane 5, N2076 RNA, isogenic wild type parent (RNase III$^+$); lane 6, N2077 RNA (RNase III$^-$); lane 7, 5' end-labeled 1.1kb *Hind*III-*Hpa*I probe; lane 8, 3' end-labeled *Msp*I fragments of pBR322 with lengths designated in nucleotides as molecular length markers. The lengths of the major transcripts are designated in nucleotides as determined by primer extension analysis (Downing *et al.*, 1990).

putative site (195 nucleotide long band) was not detected. To exclude the possibility that detection of this site was masked by the more intense transcripts, a smaller 157bp NruI probe flanking just the region of interest was used which gave only a single full length protection product under all conditions (data not shown). The results suggested that this is not an RNase E-dependent cleavage site.

Four major fragments were detected in the L1-L10 intergenic region with the 5' end-labeled BglII-HindIII probe (Figure 4). The full length protected fragment at 1257 nucleotides in length corresponds to tetracistronic transcripts encoding L11, L1, L10, and L12 initiated at the P_{L11} promoter. The bicistronic transcripts initiated at the P_{L10} promoter were detected as 810 nucleotide long fragments. The 5' end corresponding to the 660 nucleotide long band appears to result from RNase E processing since this band is absent in the RNase E^{ts} strains at the nonpermissive temperature (Figure 4, lanes 2 and 3), but is present in strains with functional RNase E activity (Figure 4, lanes 1, 4, 5, and 6). Lane 4 in Figure 4 was underloaded. An additional major band was detected at approximately 550 nucleotides in length which was detected but not characterized by Downing et al. (1990). Analysis of the RNase III mutant (Figure 4, lane 6) suggests that this site may be processed by RNase III. A 3' end was detected by Downing et al. (1990) which may correspond to one of the cleavage products resulting from RNase III cleavage. These putative RNase E and RNase III sites need to be confirmed by in vitro studies using purified RNase E and RNase III.

An additional RNase III site in the L12-β intergenic space located immediately downstream of the transcription attenuator was previously identified by Barry et al. (1980) and confirmed by Downing and Dennis (1987).
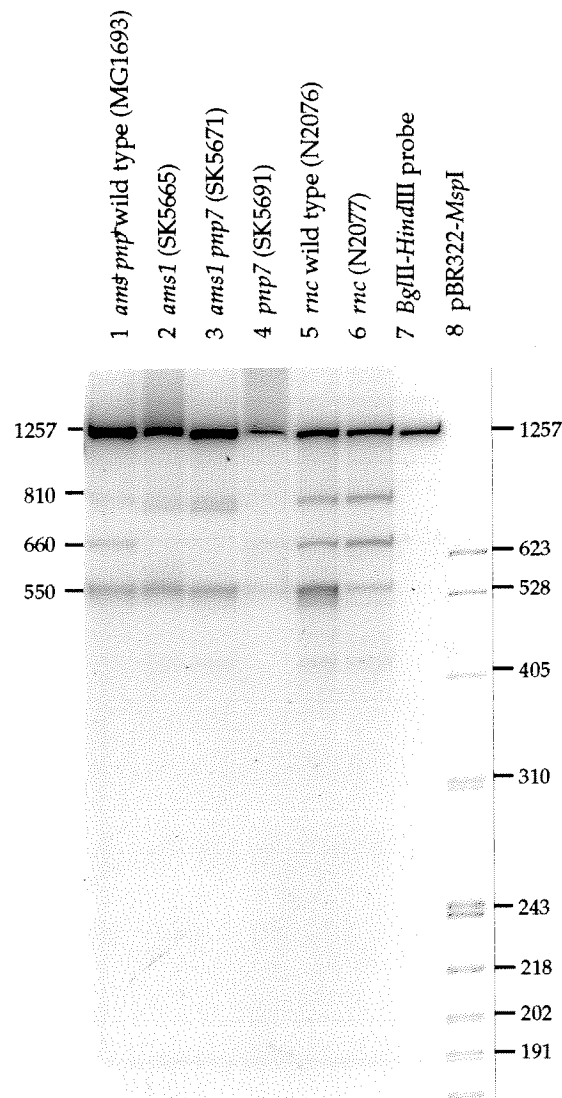
Figure 4: S1 nuclease protection analysis of *rplKAJL-rpoBC* transcripts

The 5' ends of transcripts in the L1-L10 intergenic space were analyzed by S1 nuclease protection studies using a 5' end-labeled 1.2kb *Bgl*II-*Hind*III DNA fragment. Lane designations are: lane 1, MG1693 RNA, isogenic wild type parent (RNase E$^+$); lane 2, SK5665 RNA (RNase E^{ts}) at 44°C; lane 3, SK5671 RNA (RNase E^{ts}, PNPase$^-$) at 44°C; lane 4, SK5691 RNA (PNPase$^-$); lane 5, N2076 RNA, isogenic wild type parent (RNase III$^+$); lane 6, N2077 RNA (RNase III$^-$); lane 7, 5' end-labeled 1.2kb *Bgl*II-*Hind*III probe; lane 8, 3' end-labeled *Msp*I fragments of pBR322 with lengths designated in nucleotides as molecular length markers. The lengths of the major transcripts are designated in nucleotides as determined by primer extension analysis (Downing and Dennis, 1987). Lane 4 was slightly underloaded.

To determine what effect RNase E and RNase III processing at these sites has on transcript stability in the rif region, degradation of the transcripts was monitored in the presence and absence of RNase E and RNase III activity after the addition of rifampicin. Rifampicin arrests transcription initiation while not interfering with elongation of already-initiated transcripts. Total cellular RNA was isolated from *E. coli* strains at two minute intervals for 14 minutes after rifampicin addition, hybridized to the DNA probes, and subjected to S1 nuclease digestion. Levels of *secE*, L11-L1, and L10 transcripts were probed with 5' end-labeled *PstI-BstEII* (135bp), *EcoRI-HincII* (617bp), and *PstI-HindIII* (359bp) fragments, respectively. Levels of *nusG* and β transcripts were detected with 3' end-labeled *Asp718-PstI* (218bp) and *AccI-PstI* (348bp) fragments, respectively (Figure 2). The fragments protected from S1 nuclease activity were electrophoresed on an 8% polyacrylamide gel and the resulting autoradiogram band intensities were quantified by densitometry to estimate transcript levels (Figures 5 and 6).

In the RNase E$^+$ strain (MG1693), the transcripts decayed at the same rate at 30°C and 44°C (Figure 5IA and B). In the temperature-sensitive RNase E mutant (SK5665), the decay of the transcripts at the permissive temperature closely resembled that of the isogenic wild type parent (MG1693) indicating that the *ams1* mutation does not substantially alter the normal function of RNase E at the permissive temperature (Figure 5IIA and B). However, when the RNase E mutant was shifted to the nonpermissive temperature, a dramatic stabilization of all the transcripts probed was observed (Figure 5IIA and B).

The degradation rates of the transcripts in the RNase E^{ts} PNPase$^-$ double mutant strain (SK5671), show a similar pattern to the RNase E^{ts} strain, at both the permissive and nonpermissive temperatures. The stabilization observed at 44°C was not due to the PNPase deficiency since the PNPase$^-$ single mutant

Figure 5: S1 nuclease protection assay of stability of *secE-nusG* and *rplKAJL-rpoBC* transcripts in the presence and absence of RNase E activity

Total cellular RNA from (I) MG1693 (RNase E$^+$) and (II) SK5665 (RNase E^{ts}) *E. coli* strains, was isolated at 2 minute intervals following the addition of 200 µg/ml rifampicin at 30°C (■) and 44°C (O). Twenty micrograms of RNA were used in each assay.

(IA and IIA) The *secE, nusG*, L11-L1, L10, and β end-labeled probes were hybridized to the RNA samples and subjected to S1 nuclease. The resulting fragments were run on an 8% polyacrylamide gel and visualized by autoradiography.

(IB and IIB) The autoradiograms were quantitated by a BioRad model 620 scanning densitometer and the relative band intensities were plotted logarithmically to show decay of the protected fragments over time. In all cases, the band intensities of the 14 minute samples were counted as background and were subtracted from the 0-12 minute samples and marked on the bottom axis.

I.

RNase E [+]

A.

| | secE | nusG | L11-L1 | L10 | *B* |

30C

44C

time after rifampicin addition (min)

B.

percent mRNA remaining

| secE | nusG | L11-L1 | L10 | *B* |

time after rifampicin addition (min)   (■,30C; ○,44C)

II.

RNase E [ts]

A.

B.

(SK5691) showed no change in transcript stability and resembled the wild type strain (data not shown).

In the RNase III⁻ strain (N2076), the stability of the L10 and β mRNA sequences was increased about two-fold, and the *secE*, *nusG* and L11-L1 sequences were increased about 1.25-fold relative to the isogenic wild type parent (N2077) (Figures 6I and III). These increases were much less than those observed in the RNase E temperature-sensitive strain.

As a control, the relative amounts of each of the five different S1 nuclease protection products in the seven RNA samples obtained prior to the addition of rifampicin were determined quantitatively. A constant amount of each RNA preparation (10 or 20 µg) was hybridized in parallel to an aliquot of each of the five different radiolabeled DNA probes. Since the labeled DNA is present in molar excess over the amount of complementary mRNA, the amount of S1 protection product reflects the relative amount of complementary mRNA in a 10 or 20 µg aliquot. An autoradiogram depicting the protection products is illustrated in Figure 7. The autoradiogram was scanned to quantitate the amount of radioactivity in each band and the values, normalized to the values of C600, are shown in Table 3.
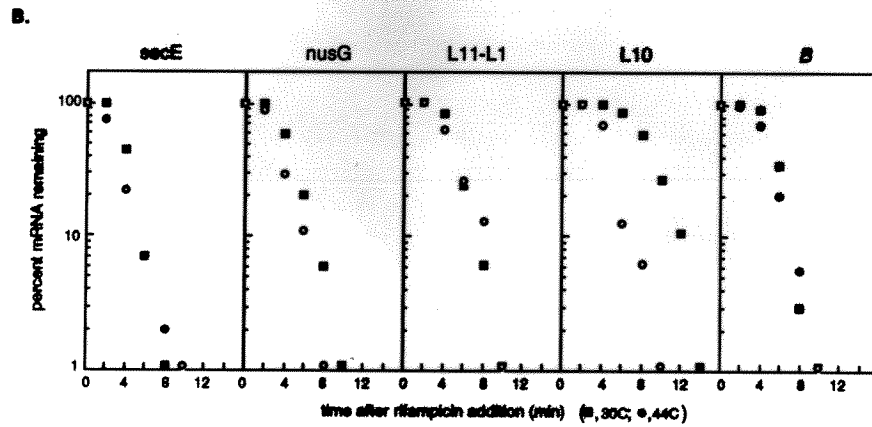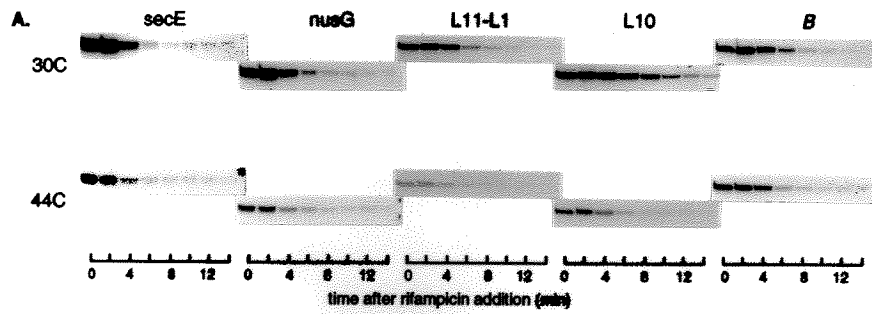
Figure 6: S1 nuclease protection assay of stability of *secE-nusG* and *rplKAJL-*
rpoBC transcripts in the presence and absence of RNase III activity

Total cellular RNA from (I) N2076 (RNase III$^+$), (II) N2077 (RNase III$^-$) and
(III) C600 (wild type) *E. coli* strains, was isolated at 2 minute intervals following
the addition of 200 μg/ml rifampicin at 37°C. Twenty micrograms of RNA were
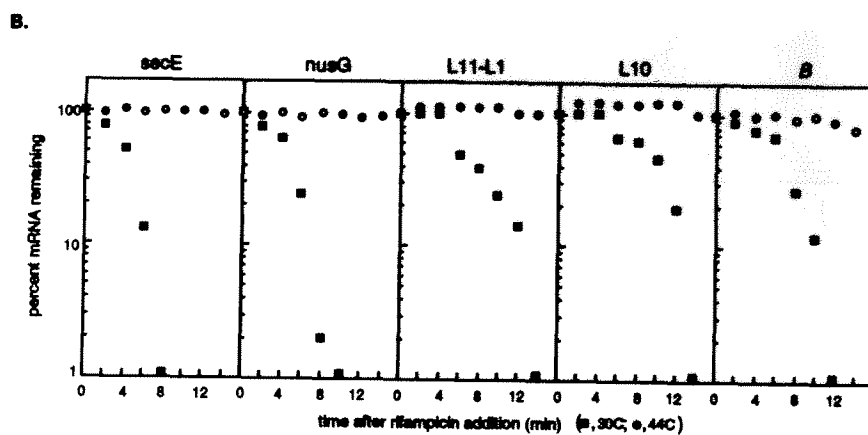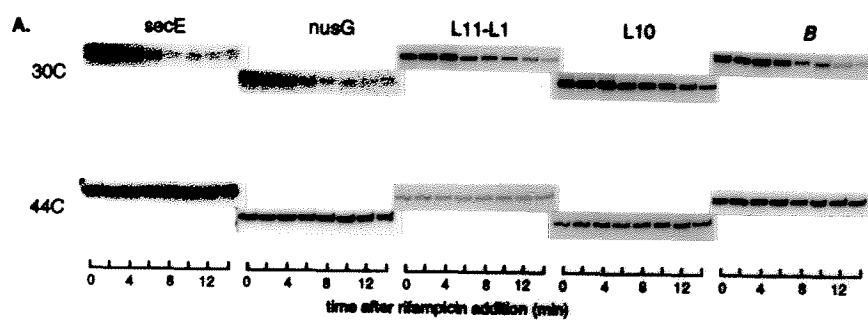used in each assay.

(IA, IIA, and IIIA) The *secE, nusG*, L11-L1, L10, and β end-labeled probes were
hybridized to the RNA samples and subjected to S1 nuclease. The resulting
fragments were run on an 8% polyacrylamide gel and visualized by
autoradiography.

(IB, IIB, and IIIB) The autoradiograms were quantitated by a BioRad model 620
scanning densitometer and the relative band intensities were plotted
logarithmically to show decay of the protected fragments over time. In all cases,
the band intensities of the 14 minute samples were counted as background and
were subtracted from the 0-12 minute samples and marked on the bottom axis.

I.

RNase III⁺

A.

| secE | nusG | L11-L1 | L10 | *B* |

37C

time after rifampicin addition (min)

B.

time after rifampicin addition (min) (■, 37C)

II.

RNase III [—]

A.

B.

III.

C600

A.

| secE | nusG | L11-L1 | L10 | B |

37C

time after rifampicin addition (min)

B.

| secE | nusG | L11-L1 | L10 | B |

percent mRNA remaining
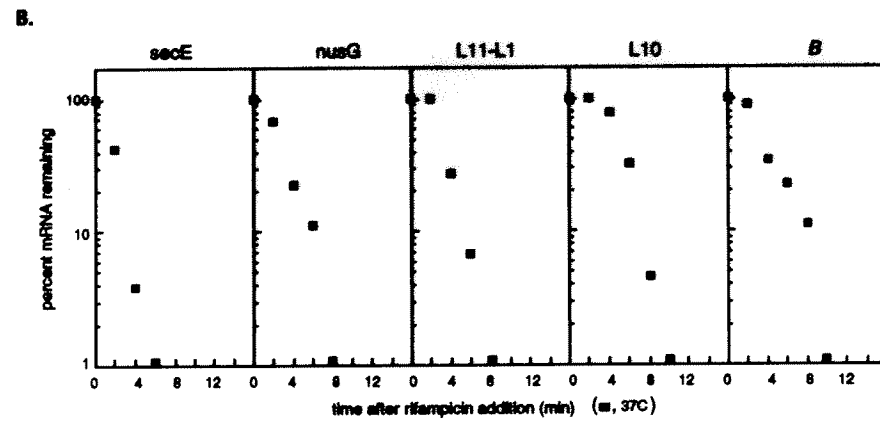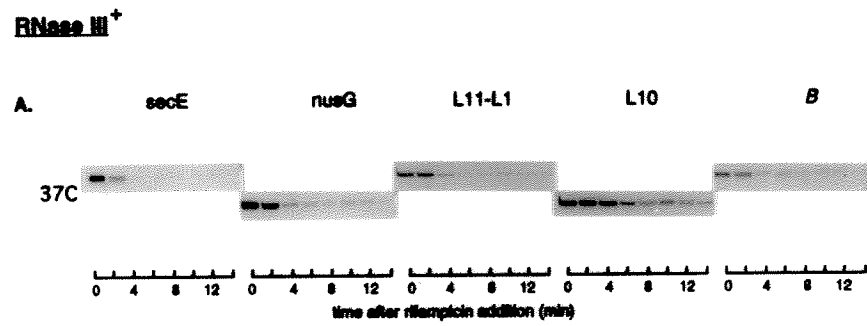
time after rifampicin addition (min)   (■, 37C)
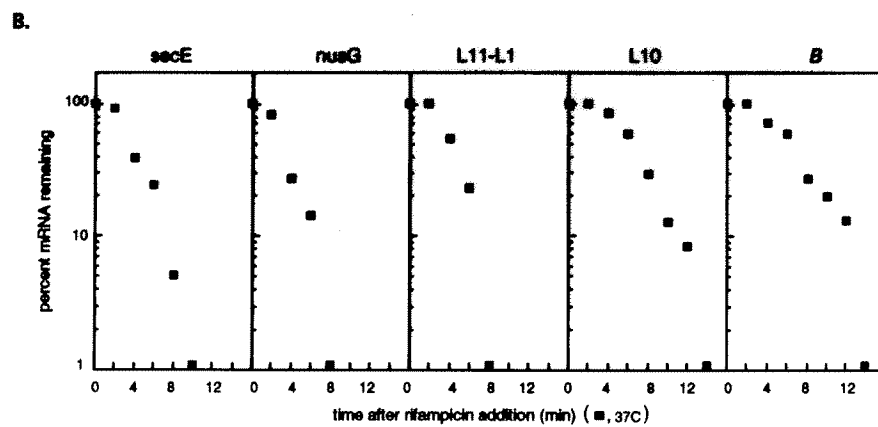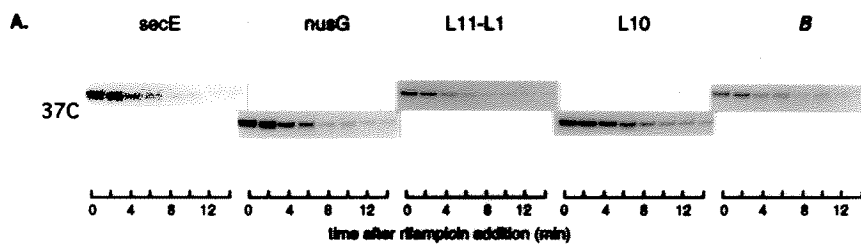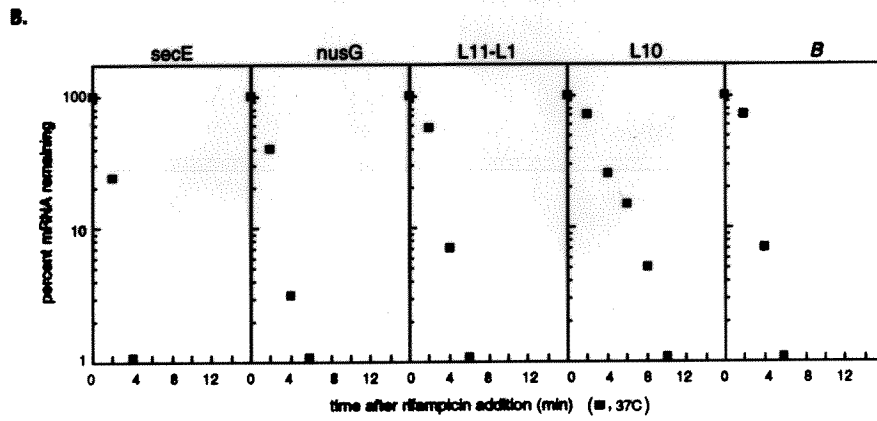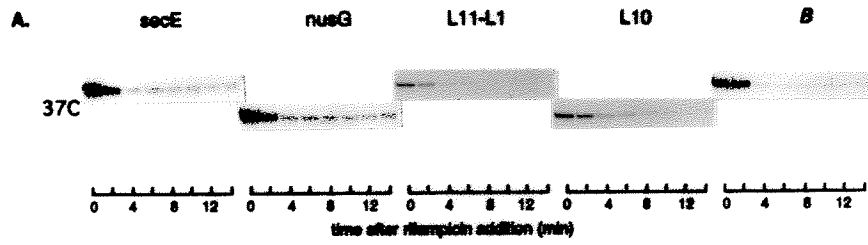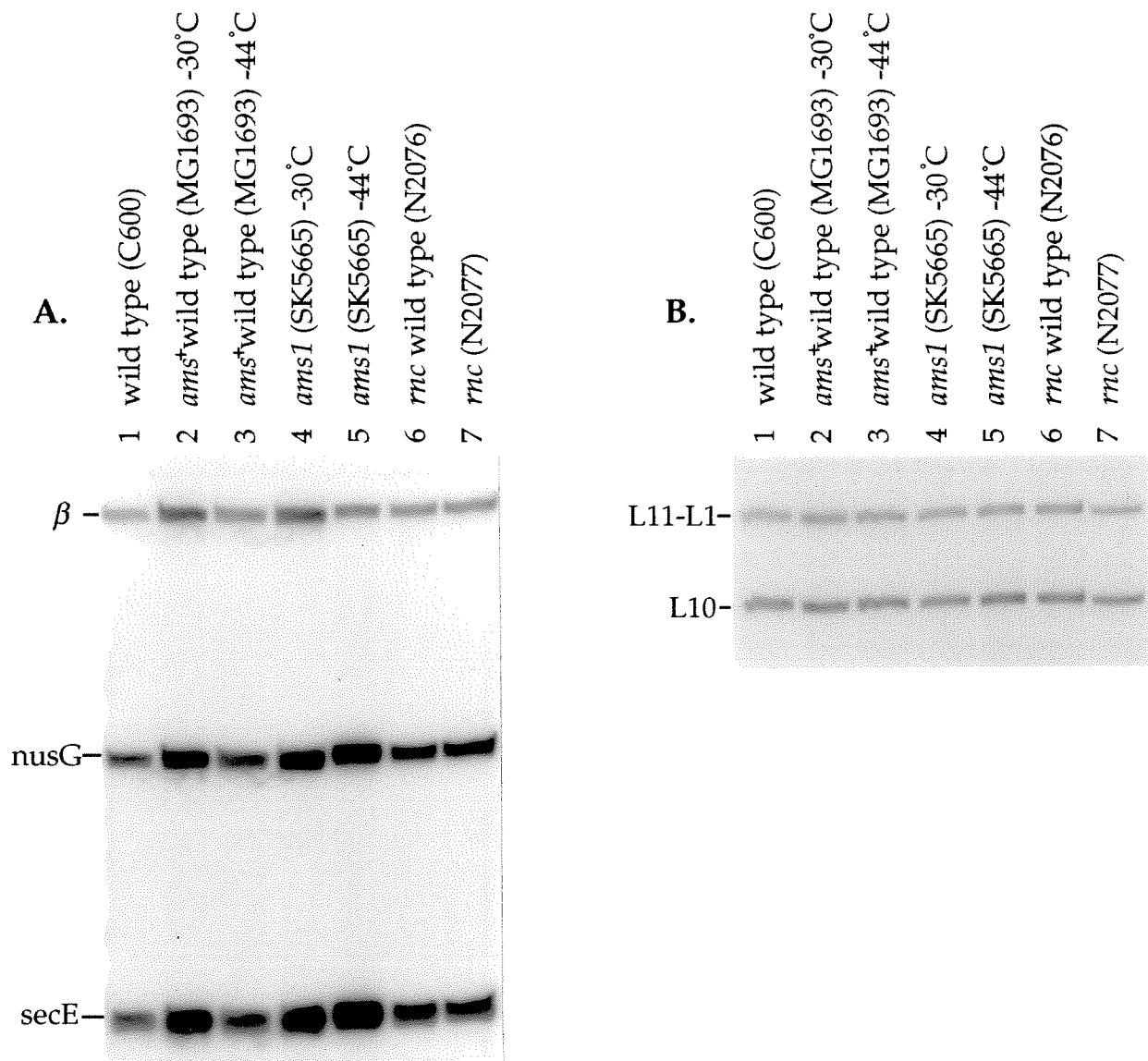
Figure 7: Relative amounts of *secE, nusG, β*, L11-L1, and L10 transcripts in various *E. coli* strains before rifampicin addition

Panel A shows the relative amounts of *secE, nusG,* and *β* transcripts at time zero after rifampicin addition. The DNA probes were hybridized to RNA (10 µg) from various *E. coli* strains. Lane designations are lane 1, C600 wild type; lane 2, MG1693 (RNase E[+]) at 30°C; lane 3, MG1693 (RNase E[+]) at 44°C; lane 4, SK5665 (RNase E[ts]) at 30°C; lane 5, SK5665 (RNase E[ts]) at 44°C; lane 6, N2076 (RNase III[+]) at 37°C; lane 7, N2077 (RNase III[-]) at 37°C.

Panel B shows the relative amounts of L11-L1 and L10 transcripts at time zero after rifampicin addition in the same *E. coli* strains as in Panel A. In this experiment, 20 µg of RNA was used for hybridization.

Probes	C600	MG1693 (ams⁺)		SK5665 (ams1)		N2076 (rnc⁺)	N2077 (rnc105)
		30°C	44°C	30°C	44°C		
secE	1.0	1.79	1.34	1.92	2.09	1.29	1.16
nusG	1.0	1.87	1.46	1.94	2.13	1.28	1.30
L11-L1	1.0	1.36	1.64	1.43	1.21	1.28	1.21
L10	1.0	1.18	1.36	1.14	1.04	1.18	1.04
β	1.0	2.23	1.54	2.31	1.42	1.19	1.19

Table 3:   Relative amounts of *secE, nusG, β,* L11-L1, and L10 transcripts in various *E. coli* strains

The relative intensities of the protected fragments shown in Figure 7 were analyzed by scanning densitometry. The values, indicative of the relative amounts of the transcripts present at time zero before rifampicin addition, were normalized to data from the wild type strain, C600. Comparisons can only be made across the rows and not between rows or within columns. The *E. coli* strains are as follows: C600, wild type; MG1693, wild type parent (RNase E⁺); SK5665, (RNaseE^{ts}); N2076, wild type parent (RNase III⁺); N2077, (RNase III⁻).

IV.   DISCUSSION

3.1   RNASE E AND RNASE III PROCESSING

The rif region of the *E. coli* chromosome provides a model system for studying endonuclease processing. Sequence analysis of the *secE-nusG* leader region reveals a potential secondary structure resembling other RNase III substrates (Figure 8). The 5' ends detected by Downing and Dennis (1987) correspond to the cleavage sites located in single-stranded bubbles on opposite sides of the stem of the proposed stem loop structure. Two RNase III sites have been identified by greatly reduced processing at these sites in an RNase III deficient *E. coli* strain.

An RNase III site may be present in the L1-L10 intergenic space. Processing at this site was reduced in the RNase III deficient strain and the sequence has the potential for forming a significant secondary structure in the RNA transcript (Figure 8). The exact site of cleavage has not been determined by sequencing or primer extension assays.

The L1-L10 intergenic space also contains an RNase E cleavage site identified by absence of processing at this site in an RNase E temperature-sensitive strain at the nonpermissive temperature. The sequence surrounding the cleavage site, GAUUU, perfectly matches the proposed RNase E consensus sequence, (G/A) AUU (A/U) (Figure 9A) (Ehretsmann *et al.*, 1992). This consensus sequence is based on a compilation of substrates cleaved *in vitro* by purified RNase E: 9S RNA, the precursor to 5S rRNA (sites a and b); RNAI, an inhibitor of ColE1 plasmid replication; and bacteriophage T4 gene 32 (sites -71 and +831) and gene 59 (site -1340) mRNA. Mutational analysis of the T4 gene 32 -71 site (Ehretsmann *et al.*, 1992) has confirmed that these nucleotides are most important for RNase E cleavage and that deviations from the consensus

42

secEnusG leader

L12-β intergenic space

L1-L10 intergenic space

5'

3'

Figure 8: Potential RNase III recognition structures in the *secE-nusG* leader, the L1-L10 intergenic space, and in the L12-β intergenic space

The sequences in the *secE-nusG* leader, the L1-L10 and L12-β intergenic spaces were analyzed for potential secondary structures. In all three regions surrounding putative RNase III cleavage sites, indicated by the arrows, the sequences could be folded into a stem loop structure characteristic of other RNase III cleavage sites. In the L1-L10 structure, the exact site of RNase III cleavage has not been determined but the approximate region is indicated by a line. The cleavage sites in the *secE-nusG* leader were determined by S1 mapping and primer extension analysis (Downing *et al.*, 1990) and the L12-β site was mapped by Barry *et al.* (1980) and Downing and Dennis (1987). The 5' and 3' ends detected in the L12-β sequence (Downing and Dennis, 1987) may be simultaneous adjacent cuts of RNase III, or the 3' end may result from rapid trimming following processing by RNase III at the 5' end-labeled site.

Figure 9: RNase E recognition sequence alignments

A) The cleavage sites of known substrates of RNase E have been aligned for comparison. The wedges indicate actual sites cut by RNase E. From 9Sa, 9Sb, RNAI, and three T4 genes, a consensus sequence has been proposed by Ehretsmann *et al.* (1992). These are the only RNase E-dependent cleavages that are cleaved *in vitro* by purified RNase E. The putative RNase E site in the L1-L10 intergenic space examined in this study was aligned. As well, the 5' end observed in the *secE-nusG* leader by S1 mapping which was thought to be an RNase E site was also aligned. This site was probably not cleaved by RNase E but by another unidentified nuclease. The exact sites of cleavage for the *secE-nusG* leader and L1-L10 intergenic sites have been determined by S1 mapping and primer extension analysis by Downing and Dennis (1987), Downing *et al.* (1990).

B) Analysis of the RNase E site in the L1-L10 intergenic space revealed a possible secondary structure downstream of the cleavage site which is also found in all of the *in vitro* substrates of RNase E. The recognition sequence is underlined.

A)

Substrate	RNase E Cleavage Site	References
9Sa 9S precursor of 5S rRNA (site a)	▼ AAUUU	Ghora and Apirion (1978)
9Sb 9S precursor of 5S rRNA (site b)	▼ AAUAA	Ghora and Apirion (1978)
RNAI ColE1 plasmid replication inhibitor	▼ UAUUU	Tomcsanyi and Apirion (1985)
T4 gene 32 (-71 site)	▼ AAUUA	Mudd et al. (1988)
T4 gene 59 (-1340 site)	▼▼ GAUUA	Carpousis et al. (1989)
T4 gene 32 (+831 site)	▼ GAUUU	Loayza et al. (1991)
CONSENSUS	(G/A) AUU (A/U)	
secEnusG leader	▼ UAGAA	this work
L1-L10 intergenic space	▼ GAUUU	this work

B)   L1-L10 intergenic space

```
               C  U  A
             C        U
               G  C
               G  C
               U  A
               C  G
               C  G
               G  C >C
               A  U
               G  C
         ▼     G  C
A A GAUUUGUUCGUU GUCGAAGAC
```
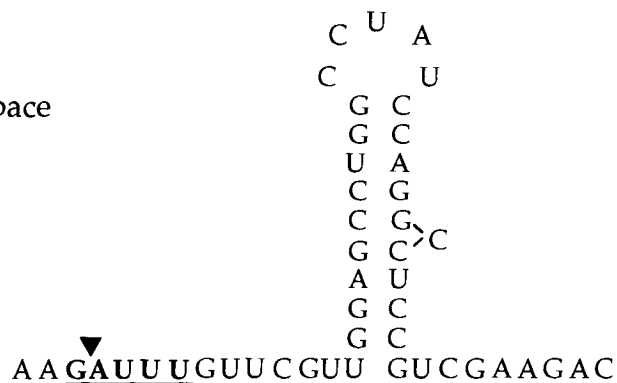
significantly reduces cleavage efficiency. In all of the above substrates, as well as the L1-L10 intergenic space, the sequences immediately downstream of the cleavage site have the potential to form a stable stem loop structure (Figure 9b). Enzymatic and chemical probing of the T4 gene 32 -71 site has confirmed the existence of this structure (McPheeters *et al.*, 1988), although the role it plays in recognition by RNase E is unknown.

Although only one RNase E site is detected by S1 mapping of transcripts from the rif region, our results clearly demonstrate that all transcripts from the region become stable in the absence of RNase E activity. Previously, Mudd *et al.* (1990b) have shown that the absence of RNase E activity increases the lifetime of bulk *E. coli* mRNA and chemically and functionally stabilizes most T4 mRNAs. In more recent studies, multiple endonuclease cleavages mediated by RNase E in the thioredoxin and S20 mRNAs, have been detected under conditions where RNase E activity has been reduced, and where PNPase and RNase II exonucleolytic activities have been eliminated (Arraiano *et al.*, 1993, Mackie, 1992). These studies suggest that RNase E cleavage events are more frequent than previously suspected or identified by cleavages *in vitro*.

The above observations suggest that not all RNase E cleavage events are equivalent in their consequences. At one extreme, some cleavages produce products, at least one of which is transiently stable and easily detected in wild type exponential phase bacteria. The RNase E mediated cleavage in the leader of the L10-L12 mRNA is an example of this; the distal product is transiently stable and easily detectable. There was no 3' end detected corresponding to the proximal product. Such cleavages might be regulatory and used to achieve differential stability and/or expression of either upstream or downstream cistrons in the mRNA. In principle, such cleavage events might be directly

controlled by features such as RNA-protein interactions, dynamic higher order structure in the RNA, or ribosome spacing or stalling during translation.

At the other extreme, many cleavages appear as random hit events on target mRNAs that are used to initiate degradation. These cleavage products are virtually undetectable in wild type exponential bacteria because they are rapidly attacked by exonucleases or other endonucleases. The intermediates can be visualized when RNase E activity is partially restricted and the exonucleases, PNPase and RNase II, are inhibited. Such degradation initiating events are random; their frequency for a particular mRNA depends directly on features such as the number of potential sites, their sequence relative to the ideal substrate, and indirectly on RNA structure and ribosome spacing. These features could easily explain the small differences in the average half-lives of different conditions of growth. Examples of this might be the multiple RNase E endonuclease events in the thioredoxin mRNA (Arraiano *et al.*, 1993).

A number of other RNA endonucleases such as RNase K, M, and I* have been partially purified and incompletely characterized (Cannistraro and Kennell, 1989, 1991, Lundberg *et al.*, 1990, Meador *et al.*, 1990, Srivastava *et al.*, 1992). The role of these nucleases in mRNA degradation has not yet been established. When RNase E activity is inhibited by temperature restriction, virtually all mRNAs are stabilized, suggesting that these additional endonucleases may function only after the degradation initiating event has been carried out by RNase E.

There is some evidence to suggest that RNase E is part of a complex or processome with other RNA processing enzymes (Jain *et al.*, 1982, Miczak *et al.*, 1991). Numerous observations suggest that RNase E is a key component in this putative complex. As stated above, only after RNase E has attached and cleaved a transcript, do the products become accessible to other potential degradative

activities. Alternatively, the temperature inactivation of RNase E may disrupt the processome complex and indirectly prevent the other components from participating in these RNase E-dependent mRNA degradation processes.

Translational regulation of the L11-L1 and L10-L12 mRNA has been studied extensively (Jinks-Robertson and Nomura, 1987, Johnsen *et al.*, 1982). In the presence of sufficient 23S rRNA, excess L1 protein binds to a target in the leader region of L11-L1 RNA and sterically prevents 30S ribosomes from binding and initiating translation. Similarly, L10 protein binds to a region about 150-220 nucleotides upstream from the L10 AUG initiation codon on the mRNA; protein binding is believed to alter the dynamic structure of the mRNA and sequester the translation initiation codon in a region of secondary structure.

No endonuclease processing site has been detected in the leader region of the L11-L1 mRNA transcript. In contrast, the RNase E mediated cleavage occurs immediately upstream of the L10 protein binding site on the L10-L12 mRNA. The relationship between RNase E cleavage at this site and translational control is not known. One possibility is that cleavage functions to balance production of L10 and L12 to L11 and L1. At least half of the transcripts from this region are tetracistronic and encode the L11, L1, L10, and L12 proteins. In this scenario, if L11 and L1 are overproduced relative to L10 and L12, the tetracistronic L11-L1-L10-L12 and bicistronic L10-L12 transcripts are cleaved by RNase E; the upstream products are rapidly degraded whereas the downstream product encoding L10 and L12 is transiently stabilized in order to restore the balance. From the site cleaved by RNase E, it is apparent that the L10 translational control site is retained intact in the downstream transcript. How cleavage would be activated by an excess of L11 or L1 protein is not clear.

Three sites cleaved by RNase III have been identified in the transcripts derived from the rif region. The first occurs in the *secE-nusG* leader, the second

occurs in the L10-L12 leader, and the third occurs in the L12-β intergenic space.

There is still no clue about the role of RNase III cleavage in the *secE-nusG* leader. In the L12-β intergenic space, the cleavage occurs within or near a sequence that is essential for efficient translation of the β cistron. It is surprising however, that the translation efficiency of β mRNA is not significantly affected by presence or absence of cleavage at these sites. This may reflect the presence of compensatory regulatory mechanisms.

Cleavage by RNase III in the other mRNA transcripts is known to have regulatory consequences. For example, RNase III cleaves its own transcript to regulate its synthesis (Bardwell *et al.*, 1989), and *E. coli pnp* expression is autoregulated through an RNase III-dependent mechanism (Robert-Le Meur and Portier, 1992).

3.2 RELATIVE mRNA AMOUNTS AND SYNTHESIS RATES

The amounts of a given mRNA sequence present in the isogenic RNase E$^+$ and RNase E^{ts} strains growing at 30°C and 44°C are directly comparable (Table 4). These relative amounts were measured by S1 mapping prior to the addition of rifampicin and are expressed relative to the amount in the RNase E$^+$ strain growing exponentially at 30°C. The measurements indicate that the amounts of the five respective mRNA sequences corresponding to the five probes from the rif region are essentially equal in the two 30°C cultures and change at most by 30% when the cultures are shifted to 44°C.

When the stabilities of the respective mRNAs are compared, a different picture emerges. First, in the RNase E$^+$ control strain, the respective mRNA half-lives change only modestly following the shift to 44°C. The largest change is for the L11-L1 mRNA where the half-life was increased from 0.75 minutes at

Probes	RNase E+						RNase Ets					
	30° C			44° C			30° C			44° C		
	$t_{1/2}$ (min)	relative amount	relative syn. rate	$t_{1/2}$ (min)	relative amount	relative syn. rate	$t_{1/2}$ (min)	relative amount	relative syn. rate	$t_{1/2}$ (min)	relative amount	relative syn. rate
secE	1	1.0	0.69	1.25	0.74	0.41	1	1.07	0.74	>20	1.16	0.04
nusG	1	1.0	0.69	1.25	0.78	0.43	1	1.03	0.71	>20	1.14	0.04
L11-L1	0.75	1.0	0.92	2	1.20	0.42	3	1.05	0.20	>20	0.89	0.03
L10	1.5	1.0	0.46	1	1.15	0.80	4	0.95	0.16	>20	0.89	0.03
β	1	1.0	0.69	1	0.69	0.48	2	1.03	0.36	>20	0.64	0.02

Table 4: Half-lives and synthesis rates of transcripts in RNase E+and RNase E ts
E. coli strains at 30°C and 44°C

The half-life of each transcript was determined from the decay curves in Figures 5 and 6 at 30°C and 44°C. The relative amounts of the five mRNA sequences were determined by S1 nuclease protection of radioactive probes by RNA isolated from cultures in the absence of rifampicin (Figure 7 and Table 3). The relative synthesis rates were calculated using the equation:

$dx/dt = X \ln2/\tau_1$, where dx/dt is the relative synthesis rate, X is the amount of mRNA relative to the amount in the RNase E strain at 30°C, and τ_1 is the half life of the mRNA sequence (see Materials and Methods).

30°C to 2.0 minutes at 44°C. Second, when the respective mRNA stabilities are compared between the RNase E$^+$ and RNase E^{ts} cultures growing at 30°C, the half-lives of the *secE* and *nusG* sequences are virtually identical whereas the half-lives of the L11-L1, L10 and β sequences are increased between two to four fold. Finally, when the RNase E^{ts} strain was shifted to 44°C, all five mRNA sequences were stabilized with half-lives greater than 20 minutes.

The above observations have several important implications. First, they suggest that even at 30°C, the *ams1* phenotype is partially manifested with respect to the stability of the L11-L1, L10 and β mRNA sequences. Second, and most important, the results indicate that both small and large changes in the stability of mRNA sequences, caused by either the temperature shift or RNase E inactivation, are balanced by a compensating change in the relative synthesis rates of the respective mRNAs. Because of these compensatory effects, the amounts of each of the five mRNA sequences remain virtually constant in the four cultures. The synthesis rates of each of the five mRNA sequences relative to the rate in the 30°C RNase E$^+$ control strain were calculated from the relative amount and half-lives of the mRNA using the first order rate equation (described in Materials and Methods, p22).

These measurements and calculations reveal a previously unrecognized phenotype for the *ams1* allele. Temperature inactivation results not only in the stabilization of mRNA sequences, but in addition, either directly or indirectly affects mRNA synthesis. That is, mRNA is not allowed to accumulate when its degradation is blocked; instead, the synthesis rate is proportionately reduced. At this point, it is premature to speculate on the mechanisms that apparently connect the amount, synthesis, and stability of mRNAs.

A number of important control experiments have already been done with the temperature-sensitive RNase E strain. First, S20 mRNA synthesis under the

control of the lac promoter is fully inducible by IPTG at the restrictive

temperature of 44°C (G. Mackie, pers. commun.). This indicates that the

transcription apparatus remains intact and functional. Second, very quickly

following the addition of rifampicin, the capacity for induction falls to zero

indicating that rifampicin efficiently blocks transcript initiation (Mudd *et al.*,

1990b). These controls imply that our observations, that RNase E inactivation

either directly or indirectly affects mRNA synthesis rates, is not artifactual.

The relative amounts of the five different mRNA sequences can also be

compared between the RNase III⁻ and its RNase III⁺ parent (Table 5). The

amounts of the respective transcripts are virtually identical in the two cultures

whereas in the absence of appreciable RNase III activity, the half-life of *secE*,

nusG, and L11-L1 mRNA sequences increase modestly from 1.0 to 1.25 minutes,

L10 mRNA increases from 1.0 to 2.0 minutes and β mRNA increases from 1.25 to

3.0 minutes. Again, although less drastic than in the RNase E^{ts} strain, the

increase in stability of the mRNA in the RNase III⁻ strain is compensated by a

corresponding reduction in synthesis rate. The fact that the coupling between

stability and synthesis rate is observed in both RNase III and RNase E deficient

strains implies that the mechanism is of a general rather than a specific nature.

	RNase III $^+$			RNase III $^-$		
Probes	$t_{1/2}$ (min)	relative amount	relative synthesis rate	$t_{1/2}$ (min)	relative amount	relative synthesis rate
secE	1	1.0	0.69	1.25	0.92	0.51
nusG	1	1.0	0.69	1.25	1.02	0.57
L11-L1	1	1.0	0.69	1.25	0.95	0.53
L10	1	1.0	0.69	2	0.89	0.31
β	1.25	1.0	0.56	3	1.0	0.23

Table 5: Half-lives and synthesis rates of transcripts in RNase III$^+$and RNase III$^-$
E. coli strains at 37° C

The half-life of each transcript was determined in RNase III$^+$and RNase III$^-$E. coli
strains at 37° C. The synthesis rate for each transcript was calculated as in Table 4.

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