NOVEL PATHWAYS FOR TRANSFER RNA PROCESSING AND MATURATION IN *ESCHERICHIA COLI*

by

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(Under the Direction of Sidney R. Kushner)

ABSTRACT

Post-transcriptional processing of all tRNA primary transcripts constitutes an important cellular activity, thought to play a role in regulation of protein synthesis and overall cell fitness. Ribonucleases are the predominant effectors of these processing events. This dissertation research aimed to achieve greater understanding of the mechanisms involved in tRNA processing, with a focus on the biological role of RNase P through microbiological, genetic and biochemical analysis. We also attempted to discern the role of polyadenylation in tRNA maturation and identify physiological interactions between RNase P and poly(A) polymerase I, the primary polyadenylation enzyme in *E. coli*.

Based on our analysis of the *valU* and *lysT* polycistronic operons, we have proposed a novel pathway for RNase P-mediated processing of tRNA primary transcripts. The enzyme works in an overall 3′ → 5′ direction, proficiently removing Rho-independent transcription terminators. Other endoribonucleases such as RNase E

play only a minor role in the processing of the large polycistrons. Dramatic reductions in the amount of mature valine tRNA do not result in a similar reduction in the level of aminoacylated valine tRNA. Furthermore, deficient processing at the 5′-ends of tRNAs does not lead to reduced levels of aminoacylated tRNAs, suggesting that a few extra nucleotides at the 5′-termini of tRNAs might not completely inhibit aminoacylation, as previously believed.

The work presented also discovered that inactivation of poly(A) polymerase I suppresses the temperature sensitivity of an RNase P mutant. There is significant polyadenylation of tRNA precursors in an RNase P mutant and removal of the polyadenylation enzyme, PAP I, results in improved processing of the tRNA precursor 3′-termini. Lack of PAP I leads to increased cellular levels of RNase T, the primary exoribonuclease involved in 3′-end processing of tRNA precursors.

INDEX WORDS: tRNA, RNase P, RNase E, valine, lysine, polyadenylation, PAP I, poly(A) polymerase I, *Escherichia coli*, *E. coli*

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DEDICATION

This dissertation is dedicated to my parents, Ashok and Alka. I am what I am and where I am because of your unconditional love, unwavering support and outstanding values.

Ma, you taught me to respect, empathize, and love.

Papa, you instilled ambition, diligence, and persistence in me. I just wish you had lived long enough to see me finish.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Ribonucleic acid (RNA) molecules form a ubiquitous group of biological polymers which were initially characterized as the carrier of genetic information of all life forms along with deoxyribonucleic acid (DNA). Since then different kinds of RNA molecules have been characterized with a variety of cellular functions. Messenger RNA (mRNA) serves as the template for mediation of the transfer of the genetic information of a cell from its DNA to proteins. Ribosomal RNA (rRNA) constitutes the catalytic component of the ribosome, the protein synthesis machinery of the cell, while transfer RNAs (tRNAs) act as adapter molecules, which recognize the genetic code during translation and transport the corresponding amino acids to the ribosome for incorporation into polypeptide chains. Small RNAs (sRNA) constitute a diverse group of RNA species that perform various functions such as regulation of gene expression, providing immunity against viruses and quality control of other cellular processes.

To survive in the wild, living cells must be equally adept at simply maintaining homeostasis or nimbly adapting and responding to their micro environment and any subtle changes therein. These cues can represent differences in availability of nutrients, variations in physicochemical conditions such as temperature, pressure, pH or presence of other organisms. While proteins and transcriptional regulation are key players in the

cellular response to environmental changes or the lack thereof, post-transcriptional processing of RNA is an essential component of a cell's capabilities to modulate its biological processes. Ribonucleases (RNase) are the primary effectors of these post-transcriptional events. Collectively, these enzymes are responsible for the processing, degradation and quality control of all different types of RNA species. Through these activities, ribonucleases can direct rapid gene expression regulation, maturation of primary transcripts, continuous recycling of ribonucleotides for incorporation into new RNA and removal of any aberrant RNA molecules or ones that are no longer required for cellular activities.

Most non-coding RNAs (all tRNAs and rRNAs as well as some sRNAs) in prokaryotes are synthesized as larger precursors that must be processed to generate the mature and functional species. This chapter provides an overview of the enzymes involved in the post-transcriptional processing of RNA molecules in the Gram-negative model organism *Escherichia coli*. Particular emphasis is given to the pathways of tRNA processing, polyadenylation and their various interactions.

ENZYMES INVOLVED IN POST-TRANSCRIPTIONAL PROCESSING OF RNA MOLECULES

Endoribonucleases

Ribonuclease E

RNase E was originally characterized as an activity whose absence resulted in increased stability of 9S rRNA precursor (*rne-3071*) and of total pulse-labeled RNA (*ams*)

at the elevated temperature of 42 °C (1-4). Subsequently, it was demonstrated that both the activities were encoded by the same structural gene, which was annotated as *rne* (5,6). The *rne* gene (24.6 min) is essential in *E. coli* and encodes a 1061 amino acid protein (7-9). Since its discovery, RNase E has been found to be required in almost all aspects of RNA metabolism. It has been shown to be responsible for tRNA maturation wherein RNase E cleaves in the intergenic regions between individual tRNAs in polycistronic transcripts and/or in the 3′-trailer sequence to remove the transcription terminator or simply shorten the trailer sequence which leaves a better substrate for exoribonucleases (10-16). RNase E is involved in the processing of 16S rRNA as well as 5S rRNA molecules from their respective precursors (1,17-24). Furthermore, it has also been shown to process M1 RNA (catalytic component of RNase P) and SsrA RNA (also known as tmRNA, responsible for recovering stalled ribosomes) at the 3′-end to remove the rho-independent transcription terminators, and plasmid maintenance RNAs such as CopA, RNAI, FinP, and Sok (25-37). Additionally, RNase E has been shown to play a major role in the biogenesis of other regulatory sRNAs as well as sRNA-mediated target decay (38-57). RNase E also acts as the central component of the degradosome, a multi-protein complex, through which it acts in general RNA decay as well as in a regulatory capacity, such as maintaining optimal ratios of FtsA:FtsZ (components of the septum forming structure during cell division) and appropriate levels of ribosomal proteins and other exoribonucleases (58-63).

The RNase E protein is composed of two functional halves (Figure 1.1). The amino-terminal half (NTH) confers the endonuclease activity and contains an S1 RNA

binding domain (RNA binding structural motif), a 5' sensor region which binds to the 5′-termini of RNA substrates, an RNase H domain whose function isn't clear, a catalytic DNase I domain, a Zn^{2+} link which binds to metal ions and a small domain which assists in holoenzyme assembly (64,65). The carboxy-terminal half (CTH) is largely unstructured with small islands capable of folding into secondary structures, and provides the scaffold for the assembly of the degradosome (66,67). The CTH contains a small phospholipid binding domain called segment A and binding sites for the other components of the degradosome, namely RhlB (DEAD-box RNA helicase), enolase (glycolytic enzyme which catalyzes the dehydration of 2-phosphoglycerate to phosphoenolpyruvate) and polynucleotide phosphorylase (PNPase, a 3′ → 5′ exonuclease) (68-73).

Other proteins have been co-purified with RNase E in sub-stoichiometric amounts and are believed to be involved in modulation of degradosome response to changing physicochemical conditions. For example, CsdA, a cold shock helicase, replaces RhlB in cells grown at sub-optimum temperatures while the chaperone protein DnaK associates with the degradosome under stress conditions (74,75). Additional proteins like poly(A) polymerase I (PAP I, the primary polyadenylation enzyme in *E. coli*) and Hfq (an RNA chaperone) have been reported to be bound to RNase E in complexes possibly distinct from the degradosome (76-78). The RNase E holoenzyme is organized as a homo-tetramer, containing a dimer-of-dimers with the interface mediated by the small domain in the NTH (79). The orientation of the four CTH subunits is

unknown and they are thought to extend out from the catalytic core (61,80). RNase E requires Mg^{2+} or Mn^{2+} cations *in vitro*, although the preferred ion *in vivo* is unknown (19).

The enzyme cleaves the substrate RNA molecules generating products with 3′-hydroxyl and 5′-monophosphate termini (19). Despite considerable efforts, a consensus sequence for recognition and cleavage by RNase E has not been definitively determined. The typical cleavage site is believed to be A/U rich and is currently defined as 5′-(A/G)N↓AU, where ↓ denotes the site of cleavage. However, the presence of nearby secondary structures or RNA-binding proteins such as Hfq can alter the cleavage site sequence specificity (35,81-87).

The 5' sensor region of the NTH of RNase E confers increased affinity for RNA substrates with monophosphorylated 5′-termini (5′-end-dependent pathway), since a phosphate pocket formed by the residues of the sensor region is too large to accommodate di- or tri-phosphorylated substrates (64,88-91). However, in an alternate mode of action, termed Direct Entry, RNase E can cleave at internal sites on certain substrates without binding to the 5′-terminus (88,92-99).

The RNase E-based degradosome is believed to be concentrated on the inner membrane, with the association with phospholipid vesicles mediated through the segment A, where several other enzymes involved in RNA processing and decay such as PAP I, RNase III, RNase P and RNase R are also reported to be localized (66,68,71,73,76,100-102). This spatial compartmentalization of the RNA processing/decay machinery away from the sites of transcription and translation is thought to play a role

in the specificity and rate of RNA processing and turnover (87,103-107). However, the importance of the localization of all these enzymes is not well understood.

Ribonuclease P

RNase P was originally identified as an activity responsible for site-specific cleavage of a tyrosine pre-tRNA at the mature 5′-end (108). Further work on the purification and properties of RNase P suggested the presence of a nucleic acid in the RNase P holoenzyme, a previously unheard of concept (108). Subsequently, the RNA component was identified as essential for the enzymatic activity and the nucleotide sequence of the gene encoding this RNA subunit (M1 RNA) was determined (109,110). A breakthrough was made when the M1 RNA subunit was identified as the catalytic component of RNase P (111).

Originally identified in *E. coli*, Ribonuclease P has since been found in all three domains of life (bacteria, archaea and eukaryotes) (112-122); with the exception of the obligatory parasitic archaeon *Nanoarchaeum equitans* (123). RNase P activity has been found to be ubiquitous and essential (*Aquifex aeolicus* and *Pyrobaculum aerophilum* were initially thought to be lacking RNase P, but an RNase P-like activity with a shortened form of the catalytic RNA was discovered in several *Pyrobaculum* species as well as in *Aquifex aeolicus*) (124-129). With few exceptions (human mitochondria, *Arabidopsis thaliana* mitochondria and plastids, and *Trypanosoma brucei* nucleus and mitochondria), the catalytic RNA component has also been found to be conserved, although more exceptions will most likely be discovered in the near future (130-133). The composition

of the RNase P holoenzyme has been found to be evolutionarily conserved to some extent as well. In bacteria, the RNase P holoenzyme comprises of a single RNA subunit and a single protein subunit; archaeal RNase P typically comprises of a single RNA subunit and four protein subunits while eukaryotic RNase P consists of at least 9 proteins along with the catalytic RNA (122,126,134-141).

RNase P catalyzes a hydrolytic reaction and cleavage of substrates generates 5'-phosphate and 3'-hydroxyl termini (142-144). The enzyme requires Mg^{2+} ions to drive the hydrolytic reaction, although other metal (II)-ions such as Ca^{2+}, Mn^{2+}, Pb^{2+}, Sr^{2+}, Zn^{2+} have been shown to allow the cleavage *in vitro* and/or *in vivo*, under various physiological conditions with varying rates of cleavage and binding affinities (145-152).

The *E. coli* RNase P holoenzyme consists of the C5 protein and the catalytic M1 RNA, which are encoded by *rnpA* (located at 83.68 min) and *rnpB* (located at 70.44 min) genes, respectively (7,110,153). Although the M1 RNA was identified as the catalytic component of RNase P based on its ability to cleave a pre-tRNA substrate in the absence of any protein cofactor *in vitro*, high Mg^{2+} ion concentrations (100 mM) had to be used to detect any enzymatic activity (111). Under physiological conditions, presence of the C5 protein subunit is essential and dramatically increases the rate of cleavage (149,154,155)

Ribonuclease G

RNase G was originally identified as a protein whose over-expression caused the bacteria to form anucleated chained cells containing long cytoplasmic axial filaments, suggesting a role in chromosome segregation and cell division (156). This protein,

originally named as CafA, was subsequently demonstrated to exhibit endonucleolytic activity and was renamed as RNase G and the gene as *rng* (73.2 min) (17,90,157-162). Sequence analysis of the *rng* gene identified extensive homology with the amino-terminal half of the *rne* gene encoding RNase E (~34% identity and ~50% similarity). RNase G is now considered to be paralog of RNase E, and members of the RNase E-G family are distributed widely through bacterial genomes (112,163-165). Unlike RNase E, the gene encoding RNase G is not essential for viability in *E. coli* (2,3,17,166).

Furthermore, the intracellular level of RNase G protein is much lower than RNase E (167,168). While it shares the preference for 5′-monophosphorylated substrates with RNase E, their cleavage site specificities do not appear to be conserved (17,64,89,159,163). RNase G participates in the processing of 16S rRNA and has been shown to act as a backup for RNase E for certain substrates (12,17,159,166,169-171). It is also believed to be involved in controlling the levels of transcripts involved in glycolysis, especially *adhE* (catalytic enzyme which functions as alcohol dehydrogenase and coenzyme A-dependent acetaldehyde dehydrogenase) and *eno* (enolase, glycolytic enzyme which catalyzes the dehydration of 2-phosphoglycerate to phosphoenolpyruvate) mRNAs (158,167,172). Overall, it is apparent that there is limited role for RNase G in *E. coli* (173).

Ribonuclease III

RNase III (encoded by *rnc*) was identified in *E. coli* as an enzyme which could specifically degrade double-stranded RNA. Since then, RNase III-like enzymes have

been found in almost all bacteria as well as in higher eukaryotes, although the composition of the conserved domains may differ. The typical bacterial RNase III enzyme consists of an N-terminal endonuclease domain (NucD) and a C-terminal dsRNA-binding domain (dsRBD). RNase III binds to stem-loop structures and generates cleavage products with 5′-monophosphate and 3′-hydroxyl termini with a 2-nt overhang at the 3′-end. The enzyme requires Mg^{2+} divalent cation for activity, although Mn^{2+}, Co^{2+} and Ni^{2+} ions have been shown to substitute resulting in altered cleavage specificity. The primary role of RNase III has been described as the maturation of 16S and 23S rRNAs from a primary 30S rRNA transcript. Furthermore, RNase III has been demonstrated to participate in the maturation or degradation of some mRNAs, tRNAs and sRNAs (174-179). Its role in decay of sRNA/mRNA duplexes is an important mechanism of gene expression regulation (180-183). Microarray analysis of the genome-wide changes in steady state RNA levels in the absence of RNase III found that ~12% of the CDS in *E. coli* were affected (184). In the same study, RNase III was suggested to regulate cysteine metabolism in *E. coli*.

Ribonuclease Z

RNase Z enzyme is required for processing of the 3′-termini of tRNA precursors which lack a chromosomally encoded CCA determinant (185-195). In various bacteria such as *Bacillus subtilis* and *Thermotoga maritima*, archaea such as *Haloferax volcanii* and *Methanococcus jannaschii*, yeast and higher eukaryotes such as *Arabidopsis thaliana*, *Drosophila*, wheat, and potato, RNase Z cleavage at the unpaired base immediately

preceding the CCA determinant generates tRNA substrates to which the required CCA motif is added by the enzyme tRNA nucleotidyltransferase (112,187,188,194-201). However, in *E. coli*, all the tRNA genes have chromosomally encoded CCA determinants (8). Therefore, the presence of an RNase Z homolog in *E. coli* was surprising. Encoded by the *rnz* gene (51.3 min), the RNase Z enzyme has been shown to be involved in degradation of certain mRNA transcripts (7,8,202). It is also required for the maturation of bacteriophage T4 encoded tRNAs, which lack an encoded CCA sequence (203). Additionally, *E. coli* RNase Z has also been shown to weakly act as a 3′ → 5′ exoribonuclease both *in vitro* and *in vivo* (204-210). The exonucleolytic activity of RNase Z (occasionally termed as RNase BN) is discussed in greater detail below.

Ribonuclease LS

RNase LS (*rnlA*, 59.6 min) was identified as an antagonist of bacteriophage T4 infection and reproduction in *E. coli* (7,8,211-213). It has also been shown to be involved to a certain extent in mRNA degradation as well as in the accumulation of a small internal fragment of 23S rRNA (212). Furthermore, it has also been suggested to play a role in the regulation of the intracellular Crp-cAMP concentration as RNase LS degrades the *cyaA* (Adenylate cyclase) mRNA transcript (214). RNase LS/RnlB has also been described as a novel toxin-antitoxin system (215). Over-expression of RNase LS reduces the stability of total mRNA; expression of RelB suppresses RNase LS activity through a direct interaction with RNase LS.

Ribonuclease I

Ribonuclease I (*rna*, 13.9 min) was the first endonuclease identified in *E. coli* and is unique in that it does not require a divalent metal ion. It remains the only identified endonuclease that generates 3'-phosphoryl-terminated RNA products (7,8,216-220). A majority (~90%) of the enzyme fraction resides in the periplasmic space (219). RNase I has broad specificity and is believed to degrade total and ribosomal RNA during periods of stress or non-growth (221-225). It has minimal role in mRNA decay (219,226). RNase I*, an altered form of RNase I, has been suggested to exist on the inner membrane and play a more prominent role in the decay of short oligonucleotides and homo-ribopolymers (227,228).

YbeY

YbeY belongs to a highly conserved family of metallo-proteins found in almost all sequenced bacterial genomes and has been described as essential in several (229-231). In fact, *ybeY* has been suggested to comprise one of the genes of the minimal bacterial genome set and was identified as essential in *Mycoplasma genitalium* genome, which has one of the smallest genomes of any organisms and has little genomic redundancy (229,232,233). While originally thought to encode a metallo-protease, YbeY has now been characterized as a single-strand specific endonuclease that plays an important role in ribosomal RNA maturation as well as in quality control of ribosomes (234). YbeY mutants accumulate precursors of 5S, 16S and 23S rRNA transcripts with immature 5'- and 3'-termini, with the defects most significant for 16S rRNA (234). The ribosomes

constituted from these defective rRNAs had increased frameshifting and read-through of nonsense codons as well as altered translation initiation factor binding (234). Furthermore, YbeY, along with RNase R, has been suggested to play a role in complete degradation of defective 70S ribosomes (235).

Exoribonucleases

Polynucleotide phosphorylase

Polynucleotide phosphorylase (PNPase, encoded by *pnp*, 71.3 min) was originally identified from the cell extracts of *Azotobacter vinelandii* as an enzyme that could catalyze the synthesis of polynucleotides from nucleoside diphosphates in a reversible reaction in the presence of Mg^{2+} (7,8,236-240). It is one of two phosphorolytic 3′ → 5′ exoribonucleases in *E. coli*, besides RNase PH (241,242).

PNPase is an important enzyme involved primarily in global mRNA decay, but with a wide variety of substrates (243,244). It has been found to be widely conserved from bacteria to plants and metazoans (245-247). Its homotrimeric ring-like complex can bind to unstructured RNA and rapidly degrade it in a highly processive manner (241,248-252). However, PNPase is strongly inhibited by RNA secondary structure (253). Consequently, it has been shown to exist in complexes with several other proteins allowing it to broaden its substrate specificity. PNPase associates with the scaffold region of RNase E along with RhlB RNA helicase and enolase, a glycolytic enzyme, to form the degradosome, which is the primary mRNA degradation machinery in *E. coli* (59,68,70,254). Furthermore, it associates with RhlB helicase in an $\alpha_3\beta_2$ structure, similar

to the eukaryotic exosome, independent of the degradosome allowing it to degrade certain RNA substrates with secondary structure (255,256).

PNPase associates with poly(A) polymerase I and Hfq (RNA chaperone) to promote polyadenylation after Rho-independent transcription terminators (257-259). Poly(A) tails have been suggested to facilitate the action of PNPase by providing an optimal binding site for initiating 3′→ 5′ degradation (260). Surprisingly, PNPase was shown to independently degrade the terminator downstream of *leuX* tRNA precursor, although this particular terminator has low thermodynamic stability (261).

PNPase has also been shown to mediate rapid target-independent degradation of small RNAs not bound to Hfq (262), exerting its effect on various global processes such as outer membrane protein synthesis (263). Interestingly, in *Bacillus subtilis*, PNPase has been suggested to play a role in DNA repair mechanisms, based on its ability to cleave ssDNA *in vitro* (264,265). However, it is unclear if such activity exists *in vivo*.

While its primary *in vivo* activity involves the degradation of RNA transcripts, PNPase has been shown to add heteropolymeric tails at the 3′-ends of mRNAs (266-268). In fact, PNPase accounts for almost all the residual polyadenylation activity in exponentially growing cells lacking poly(A) polymerase I.

PNPase mutants of *E. coli* are susceptible to cold-shock and display an increased lag phase after cold-shock treatment (269). This was found to result from PNPase mediated degradation of the mRNAs encoding several cold-shock proteins after the acclimation phase (270). They also show reduced growth rate and increased antibiotic

sensitivity (271). Furthermore, PNPase mutants accumulate defective tRNA precursors, indicating a role in quality control over the tRNA maturation mechanisms (272,273).

Multiple mutants of *E. coli* lacking other exoribonucleases along with PNPase show dramatic defects. For example, PNPase RNase II and PNPase RNase R double mutants are inviable (274,275). In the first example, accumulation of mRNA fragments 100 – 1500 nucleotides in length was observed, suggesting a deficiency in overall mRNA decay (275). In the second example, loss of cell viability was attributed to defects in the degradation of rRNAs, with accumulation of fragments of 16S and 23S rRNAs, and decrease in the amounts of ribosomes and ribosomal subunits due to defects in ribosome assembly (274,276). Furthermore, PNPase RNase PH double mutants show severe defects in ribosome assembly due to degradation or incomplete assembly of the 50S subunits (277). As a result, these double mutant cells are extremely cold sensitive, so much so, that there are very few intact 50S subunits present at the lower temperature. This suggests that the two phosphorolytic exoribonucleases, PNPase and RNase PH, drive some unique function in rRNA processing and/or ribosomal assembly that cannot be complemented by the hydrolytic exonucleases in *E. coli*.

PNPase expression in *E. coli* is subject to autoregulation mediated by the decay of its mRNA transcript (278). RNase III cleavage in the PNPase mRNA generates a 3'-end which is degraded by PNPase. PNPase levels have also been shown to be directly dependent on the level of polyadenylation of RNA transcripts (260). Furthermore, PNPase and RNase II levels in the cell have been shown to be inter-dependent (279). Recently, PNPase has been shown to respond to levels of citrate, a cellular metabolite,

suggesting a feedback mechanism between RNA metabolism and central metabolism (280).

Ribonuclease II

RNase II (encoded by *rnb*, 29 min) is a sequence independent, 3′ → 5′ exoribonuclease (7,8,220,281). It was originally identified based on its activity against poly(U) RNA, releasing nucleotide monophosphates, in a reaction which requires both divalent (preferably Mg^{2+}) and monovalent (preferably K^{+}) cations for full activity (216,282-285). Subsequently, it was found to be an important enzyme for general mRNA decay and especially RNA substrates with poly(A) tails (275,286-288).

Analysis of the crystal structure of RNase II bound to a short RNA fragment was useful in explaining several features of the enzyme which had been previously discovered (289,290). RNase II binds to its substrate molecule at two independent locations, one of which acts as a tether site and the other as the catalytic site. These results explained the observation that RNase II binds to single-stranded RNA and processively degrades its substrates (290-292). RNase II is inhibited by secondary structures because of steric hindrance in the catalytic site (253,293,294). Also, direct interactions with the ribose sugar explain why the enzyme is not able to cleave DNA (295,296). Furthermore, as the substrates become shorter in length (~12 nt), the RNA molecule can no longer be bound to both the sites and thus the enzyme becomes distributive (292,297,298). It is believed to release terminal products 3 – 5 nucleotides in length. However, RNase II can participate in the maturation of tRNAs and other stable

RNAs in the absence of other primary exonucleases (299-301). The presence of an α-helix near the amino terminus, a common membrane binding domain, in the RNase II protein explains the recent suggestion that RNase II associates with the inner membrane (302).

Besides it role in overall mRNA decay, RNase II has also been implicated in sRNA processing, regulation of the copy number of ColE plasmids by degradation of the anti-sense RNAI and in A-site mRNA cleavage after ribosome pausing and translational arrest (303-305).

While RNase II mutants show no obvious phenotype in *E. coli*, mutants lacking both RNase II and PNPase are inviable (275). Fragments of mRNAs were found to accumulate in such conditions suggesting that although they are both typically involved in mRNA decay, there is either some critical non-overlapping function or the other exonucleases in *E. coli* are not able to complement the deficiency of both these activities. Interestingly, it was found that in the absence of RNase II, the levels of about 30% of the *E. coli* mRNAs were reduced (306). This suggested that RNase II played a protective role on certain mRNAs against other nucleases. It has been suggested that either the enzyme removes poly(A) tails from the 3'-ends of mRNAs making them less suitable targets for PNPase mediated degradation or perhaps the enzyme remains bound to the substrates blocking the access of other exonucleases, especially PNPase (259,266,307-309).

RNase II expression is primarily regulated at the transcriptional level by RNase E and PNPase mediated degradation of the *rnb* mRNA (279,310,311). The Gmr protein (encoded by *gmr*, **g**ene **m**odulating **R**Nase II, located immediately downstream of *rnb*)

has been shown to regulate RNase II protein levels through some unidentified mechanism (312).

Ribonuclease R

RNase R [encoded by *rnr* (formerly *vacB*), 94.9 min] was originally identified based on its requirement for virulence in *Shigella flexneri* and *E. coli*, and its 3′ → 5′ exonucleolytic activity in strains lacking RNase II (7,8,274,313). RNase R was found to account for the residual activity against poly(A) and poly(U) substrates as well as rRNAs. RNase R is a processive enzyme and it can degrade a variety of RNA substrates, including those containing extensive secondary structures, in a reaction requiring both monovalent and divalent cations (297). The ability to process through highly structured RNA without the help of any helicases makes RNase R unique in *E. coli.* The enzyme requires a single-stranded stretch of 7 – 10 nucleotides to bind and initiate degradation, whereupon it can essentially digest rRNAs and tRNAs to completion in a highly processive manner, generating oligonucleotides 2 – 3 nucleotides in length, which are subsequently degraded by Oligoribonuclease (314). To this end, RNase R has been shown to work in tandem with poly(A) polymerase I; even if a particular RNA target does not contain sufficient residues at the 3′-end, since addition of a short poly(A) tail would allow RNase R to bind and degrade the RNA substrate (315). As such, RNase R plays a critical role in recycling of defective tRNAs and rRNAs, ensuring RNA quality control. In fact, RNase R, along with YbeY, has been suggested to play a crucial role in degradation of defective 70S ribosomes (235). Besides stable RNAs, RNase R has also

been shown to be involved in degradation of mRNA substrates with secondary structures such as those containing REP (repetitive extragenic palindromic) elements and those tagged by the SmpB/tmRNA mediated trans-translation system for degradation, which allows recovery of stalled ribosomes (315-317). Furthermore, in the absence of RNase R, the SsrA/tmRNA complex itself is not properly processed, leading to defects in trans-translation (318,319).

In *E. coli*, RNase R mutants are viable and in fact show little to no growth phenotype. However, as mentioned previously, cells deficient in RNase R and PNPase are inviable (274,276). In these strains, fragments of 16S and 23S rRNAs accumulate to high levels, defects are observed in ribosome assembly and the overall level of mature ribosomes is reduced dramatically. Whether these fragments are generated through aberrant transcription or because of defects in the ribosome assembly is not known (276). RNase R levels have been shown to increase during stress conditions such as cold shock, starvation and stationary phase (312,320-322). This increase has been attributed to increased stabilization of the RNase R protein, mediated by the global regulators RpoS and (p)ppGpp, and to differential modification of the RNase R protein in these conditions (323-325). However, the need for higher RNase R levels is not understood.

Ribonuclease T

RNase T (encoded by *rnt*, 37.2 min) is one of several 3′ → 5′ exoribonucleases in *E. coli* (7,8,326). It is unique in its ability to cleave close to double-stranded secondary structure right up to the base of the stem. Consequently, RNase T is involved in the 3′-

end maturation of tRNA precursors and other stable RNAs such as M1 RNA, 4.5S RNA, tmRNA, and 5S and 23S rRNAs (299,301,326-330). In fact, it is essential for the proper 3'-maturation of the 5S and 23S rRNA transcripts (329,330). While the enzyme has been shown to act on single-stranded DNA *in vitro*, such activity has not been characterized *in vivo* (331,332). The functional unit of the enzyme is a homodimer which requires divalent metal cations like Mg^{2+} or Mn^{2+} (333,334). RNase T has been shown to act on substrates as small as dinucleotides, however, the cleavage efficiency on substrates shorter than hexamers is poor (335). The enzyme works in a distributive manner, releasing after each catalytic event (335).

The enzyme is severely inhibited by 3'-terminal cytosine residues because of conformational changes at the active site explaining the reason why the enzyme can only remove the terminal AMP residue of a tRNA-CCA substrate (326,336,337). RNase T along with RNase PH has also been suggested to block the indiscriminate addition of poly(A) tails by poly(A) polymerase I (PAP I) to mature tRNA transcripts, which would render them non-functional (338). Over-expression of RNase T was shown to suppress the toxicity associated with increased levels of PAP I (339). While RNase T plays an important role in RNA metabolism in *E. coli*, it is not distributed widely among bacterial species (246).

Ribonuclease PH

RNase PH was originally identified as an exonucleolytic activity involved in maturation of pre-tRNAs using extracts of strains lacking several other known

exoribonucleases, in a reaction dependent on inorganic phosphate (P_i) (340,341). RNase PH (encoded by *rph*, 82.2 min) is structurally and catalytically related to PNPase in that both enzymes drive phosphorolytic cleavages using P_i as the nucleophile, generating nucleoside diphosphates as the reaction product (7,8,342,343). Furthermore, both enzymes have been found to assemble in to large hexameric rings involving a trimer of dimers (249,344-346). Also, as with PNPase, RNase PH has been shown to act biosynthetically too, adding nucleoside diphosphates to the 3'-end of RNA substrates (347). However, this activity has yet to be shown *in vivo*. Despite their similarity, their preferred substrates differ considerably. While PNPase is typically involved in mRNA degradation, RNase PH preferentially cleaves tRNA precursors, in the presence Mg^{2+}, although Mn^{2+} and Co^{2+} have also been shown to work (341,342). RNase PH prefers tRNA precursors with a few extra nucleotides after the CCA motif and is highly inhibited by tRNA-CCA and tRNA-CC substrates (342). In that regard RNase PH closely resembles RNase T. However, unlike RNase T, RNase PH is not always able to cleave right up to the base of a double-stranded stem. This observation is based on the fact the RNase PH was not able to completely process 5S and 23S rRNAs to their mature 3'-termini (329,330). Nevertheless, it has been shown to act at the 3'-end of other small stable RNAs such as 4.5S RNA and tmRNA in *E. coli* and their equivalents in other organisms (301,348,349). Recently RNase PH has also been implicated in ribosomal RNA degradation during starvation and its ability to degrade through some secondary structure has been established (350,351).

Although RNase PH is not essential in *E. coli*, certain tRNA precursors accumulate in *rph* mutants. This effect is exacerbated in multiple exoribonuclease mutants (352). RNase PH like nucleases are widely distributed across bacteria, archaea and eukaryotes; in organisms lacking RNase T, homologues of RNase PH assume additional responsibility (348,353,354). The complete nature of enzymes responsible for 3′-end processing of stable RNAs is not known in organisms lacking RNase PH homologs such as M*ycoplasma*, *Spirochetes*, *Chlamydia*, *Helicobacter pylori*, *Methanococcus jannashii* and *Halobacterium NRC-1* (246). Surprisingly, commonly used *E. coli* K-12 lab strains such as MG1655 and W3110 have a single nucleotide deletion in the *rph* gene, resulting in a frameshift which generates a shortened peptide with null activity (355).

Ribonuclease D

E. coli RNase D (encoded by *rnd*, 40.6 min) is a divalent cation dependent (preferably Mg^{2+} although Mn^{2+} and Co^{2+} have been shown to work) 3′ → 5′ hydrolytic exoribonuclease (7,8,356-359). RNase D belongs to a protein superfamily which includes both DNA and RNA exonucleases (246). Its homologs have been found in very few bacteria, such as *Proteobacteria* and *Mycobacteria*, but in almost all eukaryotes (246). In *E. coli*, RNase D is highly specific for tRNA molecules and a few other stable RNAs (301,360,361). It prefers tRNA precursors with some extra nucleotides following the –CCA motif or tRNAs with damaged 3′-ends (328,358). While its activity on tRNA precursors suggests a distributive mode of action, its ring-shaped architecture, discerned

from a crystal structure, suggests a more processive mechanism (358,362). Its activity is highly inhibited by RNA secondary structure.

RNase D mutants show no phenotypic or growth defects and is only essential in mutants lacking other exoribonucleases such as RNases II, BN, T and PH (363,364). In this scenario, RNase D was able to complement the other exonucleases by processing tRNAs and other stable RNAs such as 4.5S RNA and tmRNA, albeit to a small extent (299,300). In *E. coli*, RNase D expression is regulated at the translational level by several mechanisms such as the use of UUG as the translation initiation codon, the presence of a transcription terminator like stem-loop structure and a stretch of eight uridine residues between the promoter sequence and initiation codon that affect ribosome binding to the *rnd* message as well as the usage of several rare codons in its peptide sequence (365-367). However, the implications of this extensive regulation and its primary role in *E. coli* have yet to be determined.

Ribonuclease BN

As mentioned above, RNase Z has also been demonstrated to act as a 3′ → 5′ exoribonuclease. This activity of RNase BN was originally identified based on its ability to remove the terminal nucleotide from the 3′ end of a synthetic tRNA substrate (204). Subsequently, it was demonstrated the RNase BN was sufficient to sustain strains lacking the other exoribonucleases, RNases T, PH, II and D (300,364). This suggested significant functional overlap between the various exonucleases. The gene encoding RNase BN was identified to be the same gene encoding the previously discovered

RNase Z (205,207). Recent work studying the *in vivo* role of RNase Z/BN suggests that although the enzyme primarily acts as an endoribonuclease, it can act as a weak exoribonuclease in the absence of other dominant exoribonucleases (202,208-210,368).

Oligoribonuclease

Oligoribonuclease (Orn), encoded by *orn*, mapping at 94.6 min, is a 3′ → 5′ hydrolytic exoribonuclease, unique in its ability to degrade short RNA oligonucleotides down to mononucleotides (7,8,369-372). RNA fragments 2 – 5 nucleotides in length are generated from degradation mediated by PNPase, RNase II and RNase R because of their inability to bind to such short substrates (373). In the absence of Orn, these fragments accumulate and are considered deleterious because of their ability to hybridize to transcription initiation bubbles and inhibit transcription (373-375). Orn assembles as a homodimer and mediates its cleavage using Mn^{2+} cation, although Mg^{2+} has been shown to work with reduced efficiency (369,376). The enzyme is highly processive in its activity, with higher affinity for 5-mer oligoribonucleotides than smaller substrates, although the reaction rate decreases with the increasing chain length (370). The enzyme requires a free 3′ -OH end, but is not sensitive to the phosphorylation state of the 5′-terminus. While homologs of Orn have been found in several bacteria, such as *Proteobacteria* and *Actinomycetes*, all eukaryotic genomes and even in a member of *Entomopoxvirinae* subfamily (*Diachasmimorpha longicaudata entomopoxvirus* DlEPV), they are absent in many bacterial clades and in all archaea, suggesting that other proteins can perform the role of Orn in these organisms (246,377,378). For example, in *B. subtilis*, the

combination of RNase J1 (5′ → 3′exoribonuclease), NrnA and NrnB (phosphodiesterases) is believed to perform the function of Orn (379-382). NrnC, a functional homolog of Orn with some sequence similarity to RNase D, has been identified in several *Alphaproteobacteria* and *Cyanobacteria* (383). Furthermore, while Orn is the only exonuclease essential in *E. coli*, its homologs in some other bacteria, such as *Pseudomonas* and *Streptomyces* can be deleted, suggesting redundancy in oligoribonuclease activity (373,375,378,384,385).

Other Enzymes Involved In RNA Metabolism

Poly(A) polymerase I

Poly(A) polymerase I (PAP I, encoded by *pcnB*, 3.4 min) is the primary polyadenylation enzyme in *E. coli*, referring to the universal process of untemplated addition of adenylate residues at the 3′-ends of RNA molecules (7,8,386,387). Polyadenylation was first discovered in bacteria in the early 1960's and the *E. coli* poly(A) polymerase was the first such enzyme identified and purified (388-391). In eukaryotes, poly(A) tails are primarily involved in transcription termination and in protecting messenger transcripts from degradation by exoribonucleases. In contrast, polyadenylation in prokaryotes has been shown to destabilize transcripts and target the RNA substrates for degradation (30,392-398). PAP I, in collaboration with the endo- and exo-ribonucleases, has been shown to have a role in the decay of important mRNAs such as *lpp*, *trpA*, *rpsO*, *crp*, *rho*, *and lacZ* (396,399-408). The absence of PAP I leads to stabilization of the mRNAs and also the decay intermediates generated through

endonucleolytic cleavages (398,402,409-414). Furthermore, it has been observed that although secondary structures such as stem-loops inhibit PNPase/RNase II-mediated exonucleolytic degradation, repeated switching between polyadenylation and nucleolytic activities of PAP I and PNPase/RNase II, respectively, allows the degradation of stable structures present at 3'-ends of primary transcripts and their decay intermediates (259,293,415-417). It has also been proposed that PAP I and the exoribonucleases continuously compete for the available 3'-ends of mRNA molecules, thus regulating the overall level of polyadenylation in the cell, as well as the typical length of the poly(A) tails (415).

In wild type cells, polyadenylation is a very common phenomenon (>90% of the ORF's in *E. coli* are polyadenylated to some extent) and the tails are usually 5 – 40 nucleotides in length (401,418,419). Although originally thought to be limited to mRNAs, polyadenylation has now been detected on almost all RNA species including rRNAs, tRNAs and sRNAs. While the polyadenylation of the stable rRNA and tRNA substrates is believed to be a quality control mechanism to assist in degradation of immature and defective molecules, polyadenylation-mediated control of different sRNA levels is an important and integral mechanism for regulating gene expression of their targets (12,30,34,36,182,257,261,272,418,420-425).

Despite its broad substrate specificity, PAP I levels are fairly low (only ~40 molecules of PAP I are estimated per cell in *E. coli*) (257,401). This level is kept low through a combination of factors including low level of transcription from the promoter, presence of a weak non-canonical translation initiation codon (AUU), degradation of the

pcnB message by PNPase and growth stage dependent regulation (401,418,426-428). Over-expression of PAP I resulted in several hundred fold higher level of polyadenylation, longer poly(A) tails, rapid cell death and lethality (418). This toxicity was proposed to be associated with significant polyadenylation of 23S rRNA in strains over-expressing PAP I, targeting it for degradation. However, recent work has demonstrated that over-expression of PAP I leads to indiscriminate polyadenylation of tRNAs, which are typically highly stable molecules and poor substrates for polyadenylation, making them unavailable for aminoacylation. Consequently, there is a dramatic reduction in the fraction of aminoacylated tRNAs available for protein synthesis, resulting in cell death (338,339). This effect was highly magnified in the strains lacking various exoribonucleases involved in tRNA processing.

RppH

For a long time it was presumed that endonucleolytic cleavages were the initial and rate limiting step in mRNA degradation (14,106,429). However, it was also known that RNase E activity is 5′-termini dependent, and is greatly enhanced on 5′-monophosphorylated substrates compared to triphosphorylated ones (88,430,431). Subsequently, RppH (RNA pyrophosphohydrolase) was discovered as the enzyme that was responsible for removing a pyrophosphate moiety from the 5′-termini of several triphosphorylated RNA substrates in *E. coli*, converting them into more labile monophosphorylated substrates, thus accelerating their decay by the 5′-end-dependent degradation enzymes, primarily RNase E (95,432). In the absence of RppH, over 300

transcripts were found to be stabilized in *E. coli*, suggesting a wide array of substrates, although recent work by Bowden & Kushner (unpublished data) suggests that the actual number might be considerably lower. While it was originally proposed that pyrophosphate removal from the 5'-end was the actual rate limiting step for the mRNA decay, it has since been found to be not true (433). Instead, for certain RppH substrates, the rate limiting step precedes the triphosphate removal. Furthermore, RppH activity is inhibited by the presence of stem-loop structures at the 5'-termini of substrates. In *B. subtilis*, a requirement for at least 2 and preferably 3 or more unpaired nucleotides at the 5'-end was shown for optimal RppH activity (434). The presence of another pyrophosphate removing enzyme has also been suggested. RppH homologs have been found in several other bacteria, suggesting that this process is widely conserved (435-439). In eukaryotes, an equivalent process to remove the 7-methylguanylate cap (m^7G) from mRNA substrates exists (440-442).

Hfq

Hfq (encoded by *hfq*, 94.8 min) is a small and abundant RNA binding protein, which was originally identified based on its requirement for the replication of the (+)ssRNA virus Qβ (7,8,443). The absence of Hfq was found to result in wide ranging pleiotropic defects such as decreased growth rate, increased cell size, osmosensitivity, and increased sensitivity to ultraviolet light because of its requirement for translation of the σ^s subunit of RNA polymerase (444-447). Its potential role in poly(A) mediated mRNA decay was indentified when inactivation of the *hfq* gene resulted in reduction of

the length of poly(A) tails synthesized at the 3'-end of the *rpsO* mRNA by PAP I (448,449). Hfq is now understood to modulate the level of poly(A) as well the length of poly(A) tails in the cell through its physical association with PAP I and PNPase (53,76,257). Furthermore, PAP I mediated polyadenylation of mRNAs containing Rho-independent transcription terminators is greatly reduced in mutants lacking the Hfq protein (257). Also, the presence of Hfq increases the processivity of the PAP I enzyme (450,451).

Hfq has also been shown to play an important role in sRNA processing and decay as well as sRNA mediated target decay, where it can promote the binding of RNase E to RNA substrates resulting in faster degradation, or it can bind to the A/U rich sites which would otherwise serve as RNase E cleavage sites, thus protecting a particular substrate from RNase E mediated decay (40,48,53,452,453).

POST-TRANSCRIPTIONAL PROCESSING OF RIBOSOMAL RNA

The *E. coli* genome contains seven ribosomal RNA operons (*rrnA, rrnB, rrnC, rrnD, rrnE, rrnG, rrnH*) which encode large 30S rRNA primary transcripts (8,454). All the operons include genes encoding 16S, 23S and 5S rRNAs as well as tRNAs. The operons may differ in the number of 5S rRNA genes (*rrnD* encodes two) as well as in the number and identity of the tRNA genes (E.g. *rrnB*, *rrnE* and *rrnG* each contain one glutamate tRNA while *rrnC* contains glutamate, aspartate and tryptophan tRNAs). The 30S precursors must undergo post-transcriptional processing to generate the individual mature rRNA species. It is presumed that before processing initiates, the primary

transcript undergoes folding, modification and binding of several ribosomal proteins (455,456).

The first step in ribosomal RNA processing involves RNase III mediated cleavages at the stem-loops of 16S and 23S rRNAs generating pre-16S (17S), pre-23S (25S) and pre-5S (9S) precursors (457). Each of these precursors requires further processing at both termini to generate the mature rRNA species. The pre-16S precursor is processed by RNases E and G to generate the mature 5′-termini (17). The maturation of the 3′-termini of 16S rRNA is not completely understood. It has been proposed that YbeY along with RNase R and/or PNPase is responsible for the 3′-end processing (234,235). RNase E also cleaves the 9S precursor within a few nucleotides of both the 5′- and 3′-termini of mature 5S rRNA (1,23). RNase T is responsible for maturation of the 3′-termini of both 5S and 23S rRNAs (329,330). The enzymatic activities responsible for 5′-end processing of both pre-23S and pre-5S precursors are unknown (458,459).

PATHWAYS OF mRNA DECAY

While ribosomal and transfer RNAs are highly stable molecules that can function across several generations, mRNAs are inherently unstable. Indeed, the half-lives for most mRNAs have been determined to be between 60 – 120 seconds, although messages with half-lives as long as 15 min have been observed (93). This metabolic instability is integral for the post-transcriptional modulation of gene expression, facilitating adaptability of an organism, as well as to ensure continuous recycling of ribonucleotides (460-463). A number of factors including the primary sequence, secondary and tertiary

structures, translational efficiency, transcriptional and translational repression, presence of 5′-triphosphate and 3′-poly(A) tail, and binding of other proteins or RNAs can contribute to the overall stability and lifetime of an mRNA species (94-96,106,396,398,432).

Once an mRNA molecule is designated to be degraded, a key step in the initiation of decay is an initial endonucleolytic cleavage (Figure 1.2). In *E. coli*, RNase E is the enzyme primarily responsible for the first cleavage (3,464,465). Occasionally, other endoribonucleases such as RNase G and RNase P have been shown to catalyze the initiating cleavage (173,202,212,466,467). Nevertheless, once cleaved, degradation proceeds rapidly to completion, through cooperation by exoribonucleases and other enzymes such as Poly(A) polymerase I, Hfq and RNA helicases. Depending on the substrate, RNase E can bind and cleave the substrate in either a 5′-end-dependent manner or alternatively, using the direct entry pathway (88,99). RppH converts the 5′-triphosphate into a monophosphate for the 5′-end-dependent pathway (432). Multiple endonucleolytic cleavages result in several short fragments which can be degraded from the 3′-end exonucleolytically or undergo further endonucleolytic cleavages. PNPase and RNase II are the primary exonucleases involved in the degradation of long single stranded RNA fragments (275,468). Addition of long poly(A) tails by PAP I targets these small fragments for degradation by PNPase and RNase II (266,306,398). RNase R can efficiently process through any secondary structure such as stem-loops (297,315). Additionally, repeated alternating polyadenylation by PAP I and exonucleolytic degradation by PNPase/RNase II can also process secondary structures with some

efficiency (259). Most of the exonucleases in *E. coli* cannot process RNA substrates down to mononucleotides. Consequently, the resulting 2 – 5 nucleotide fragments are further processed by Oligoribonuclease to generate mononucleotides (369,372).

PATHWAYS OF POST-TRANSCRIPTIONAL PROCESSING OF tRNAS

In *E. coli*, the 86 tRNA genes are often organized in polycistronic transcripts containing genes encoding other tRNAs, rRNAs and also mRNAs (8,185). These tRNA transcripts are almost always synthesized with 5′-leader and 3′-trailer sequences, which must be removed post-transcriptionally to convert the primary transcripts into mature and functional tRNAs (469,470). While the 5′-processing activity was identified as RNase P and studied in great detail (471), the processing of the 3′-termini was more difficult to identify because of the role of endonucleolytic activity as well as multiple exonucleases (299-301,328,364,472,473).

In the first set of analyses of processing of polycistronic transcripts, RNase E was identified as an important endoribonuclease contributing to the initial processing of multimeric tRNA transcripts (10,11). Consequently, a model for tRNA maturation was proposed (Figure 1.3) wherein RNase E cleavages in the intergenic regions between tRNAs serve to separate the individual precursors as well as remove any Rho-independent transcription terminators. These pre-tRNAs are then processed at the 5′-end by RNase P and at the 3′-termini by a combination of exoribonucleases including RNase T, RNase PH, RNase II, RNase D, PNPase and RNase BN (11). The specific enzyme(s) involved are believed to depend on the length of the 3′-trailer remaining. For

long trailer sequences, PNPase and RNase II initially remove several nucleotides releasing precursors with short 3′-trailer sequences, which are then processed primarily by RNase T and RNase PH. On the other hand, RNase T and RNase PH can efficiently remove any short 3′-trailers directly. Furthermore, RNase E was proposed to be responsible for processing and separation of all the polycistronic tRNA precursors (10,11).

Recently, another pathway which involves direct RNase P cleavages at the mature 5′-termini of the different tRNAs in a polycistronic transcript was discovered for certain transcripts (474). This model (Figure 1.4) eliminates the requirement for RNase E activity to first separate the tRNAs into their individual precursors. Subsequently, variants of this model were identified which differ in the removal of the Rho-independent transcription terminator and the 3′-termini processing (12,261). Overall, it is now understood that the pathways for processing of tRNA precursors are highly diverse.

RNase P has been historically considered as essential because of its irreplaceable role in the 5′-end maturation of all tRNA precursors. However, based on the RNase P model of tRNA processing (Figure 1.4), we hypothesized that RNase P could instead be essential because of its role in the 5′-end maturation of a specific tRNA, rather a general involvement in the processing of all tRNA precursors. We proposed a scenario wherein the absence of RNase P results in incomplete processing of all copies of a tRNA, resulting in severe shortage of the mature and functional form of that tRNA. Consequently, protein synthesis would be slowly shutdown, ultimately leading to cell

death. In such a situation, ectopic synthesis of a mature form of that tRNA would bypass the requirement for RNase P, making it no longer an essential activity. To prove our hypothesis, we conducted thorough sequence analysis of all tRNA genes in *E. coli* and concluded that valine tRNA was the most likely candidate for which all the transcripts encoding $tRNA^{val}$ required RNase P mediated processing. We studied the processing of two polycistronic operons containing five valine tRNAs and identified a novel tRNA processing pathway involving RNase P. This work is presented in Chapter 2.

Polyadenylation was traditionally assumed to be an activity limited to mRNAs and aberrant RNA transcripts. The long and largely unstructured poly(A) tails added to the 3′-ends of target substrates allow better binding of the 3′ → 5′ exonucleases including RNase II, PNPase, and RNase R. This ensures efficient turnover of mRNA molecules and any defective transcripts, including aberrant stable RNAs such as ribosomal RNAs. However, recently it was demonstrated that even tRNAs and other stable RNAs that are not defective are actually polyadenylated frequently. This observation was suggested to constitute a novel regulatory mechanism wherein the 3′-end processing activities of RNases T and PH continuously compete with the polyadenylation activity of PAP I to ensure rapid processing of the 3′-ends of those RNA precursors. In a fortuitous discovery, we found that inactivation of PAP I suppresses the conditional lethality associated with the inactivation of RNase P temperature sensitive mutants. Aside from over-expression of catalytically active M1 RNA, this constitutes the first evidence of extragenic suppression of an RNase P mutant. While RNase P is primarily involved in processing events at the 5′ termini, polyadenylation would only

occur at 3'-termini of any RNA substrate. Therefore the observation that a mutant strain deficient in RNase P and PAP I survived at the non-permissive temperature was very intriguing. We decided to study the possible interaction between RNase P and PAP I activities. This work also led us to explore the biological significance of RNase P and the niceties of tRNA charging pathways. We identified several novel features of RNase P processing of tRNA precursors, tRNA aminoacylation, and PAP I-mediated regulation of cellular exoribonuclease levels. This work is presented in Chapter 3.

Overall, my research has focused on acquiring a better understanding of the mechanisms and participants of post-transcriptional events in the model bacterium *Escherichia coli*. We have identified several new features of processing of tRNAs from their primary transcripts. Next, we discovered the first instance of extragenic complementation of an RNase P mutant. Our work also provides better understanding of tRNA aminoacylation pathways. In summary, our work provides important insights into the physiological roles of RNase P and Poly(A) polymerase I.

FIGURES

Figure 1.1: Model diagram of RNase E

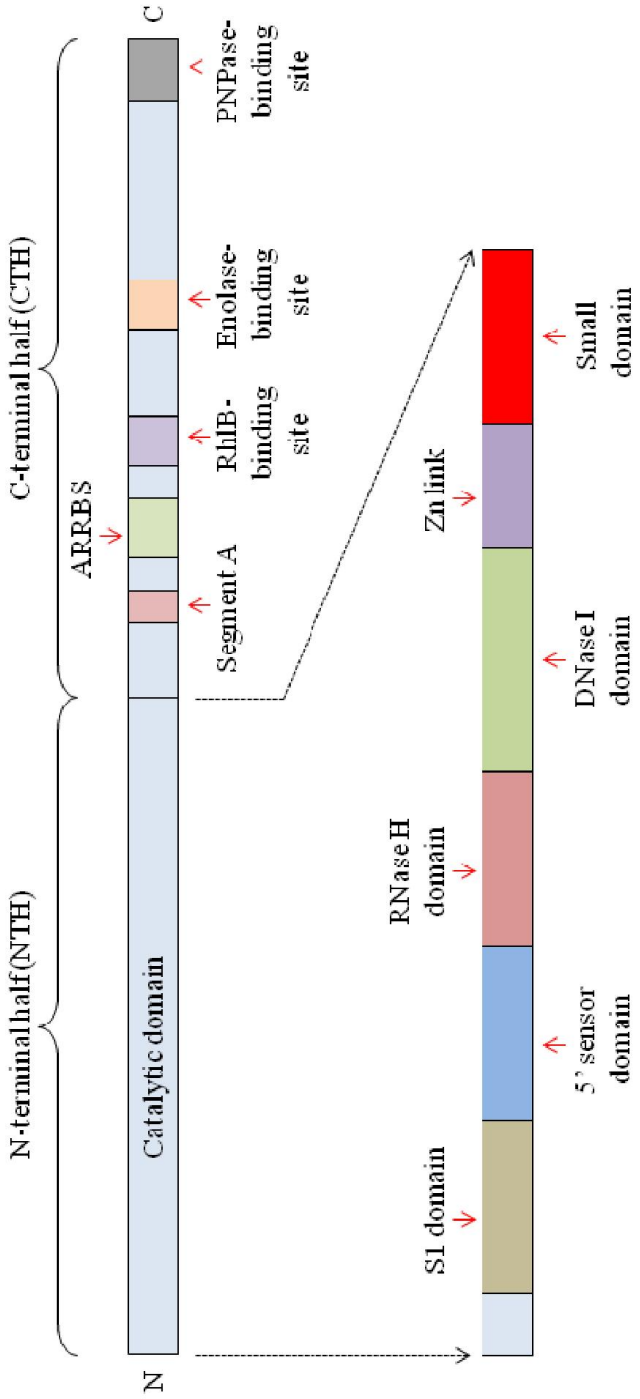


Figure 1.2: Pathways of mRNA decay in *Escherichia coli*

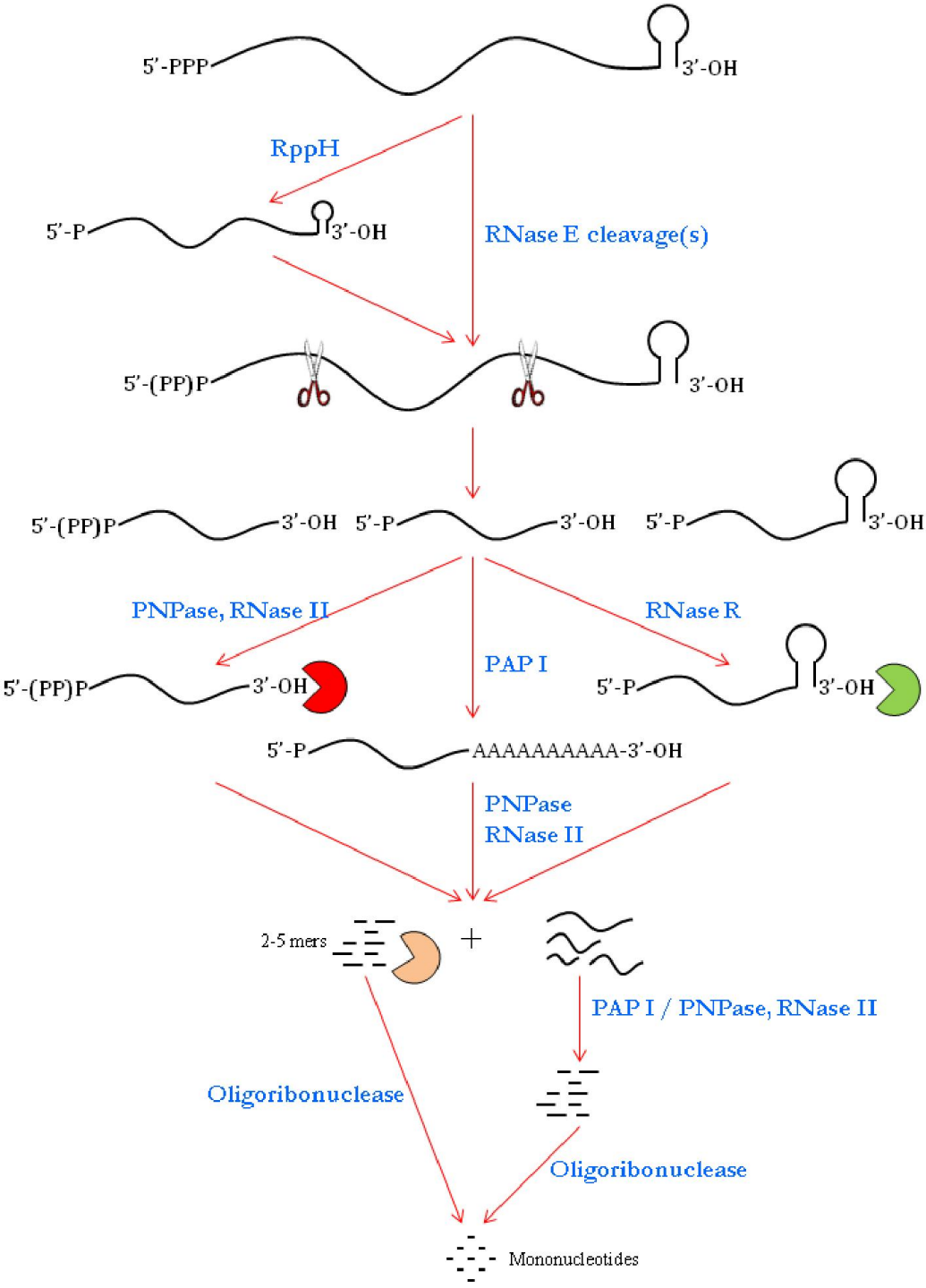


Figure 1.3: RNase E-dependent model of tRNA processing

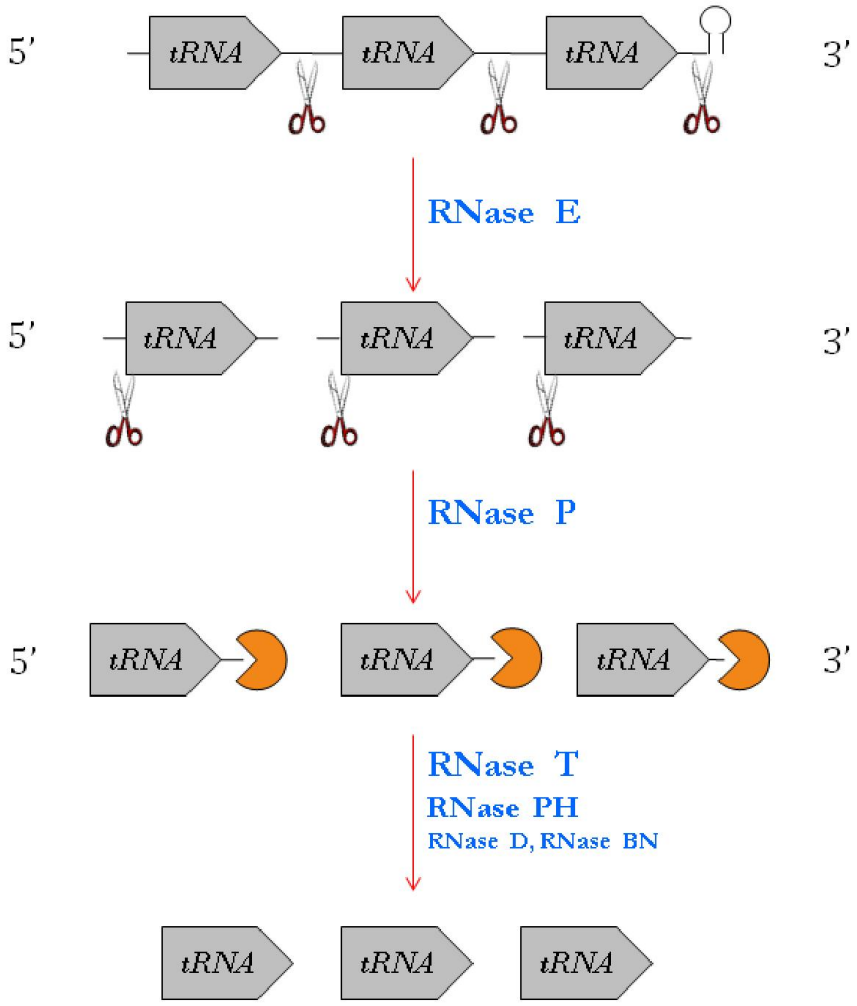
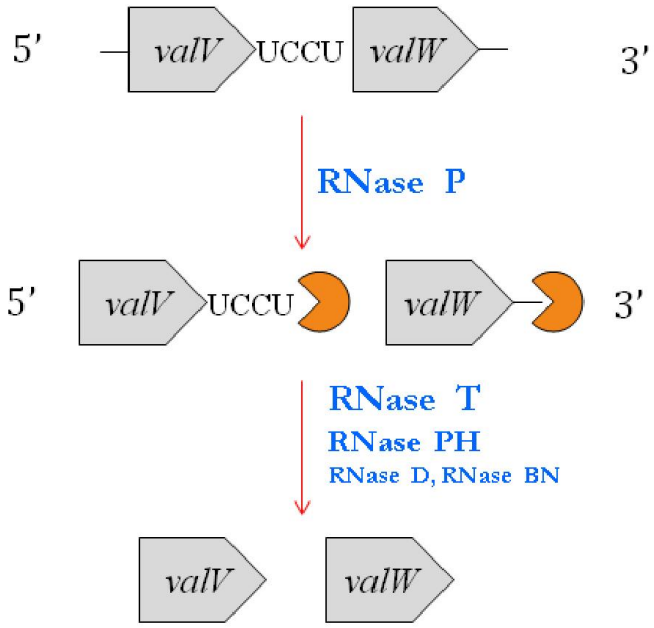


Figure 1.4: RNase P-dependent model of tRNA processing



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CHAPTER 2

RNASE P IS REQUIRED FOR THE INITIAL PROCESSING OF ALL SEVEN VALINE tRNAS IN *ESCHERICHIA COLI*[1]

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ABSTRACT

Here we report that RNase P is required for the initial separation of all seven valine tRNAs from three distinct polycistronic transcripts (*valV valW*, *valU valX valY lysY* and *lysT valT lysY valZ lysW lysZ lysQ*). In the absence of RNase P the level of mature valine type I tRNAs decreases over 30-fold. Particularly significant is the mechanism by which RNase P processes the large polycistronic transcripts. Specifically, the enzyme appears to initiate degradation by first removing the Rho-independent transcription terminators for the *valU* and *lysT* transcripts. Subsequently, it proceeds in the 3' → 5' direction generating one pre-tRNA at a time. Although it has been long thought that the essential function of RNase P in *E. coli* was to generate the mature 5' termini of all 86 tRNAs, we demonstrate here that failure to mature the 5' termini of all seven valine tRNAs only results in an ~2-fold reduction in the level of their aminoacylated species. Surprisingly, RNase E only plays a very minor role in the processing of all three valine polycistronic transcripts.

INTRODUCTION

The *E. coli* genome contains 86 transfer RNA (tRNA) genes that are organized as either monocistronic transcripts or complex operons, containing other tRNAs, messenger RNAs (mRNA) or ribosomal RNA (rRNA) genes (1,2). Every tRNA is initially transcribed as a precursor with additional nucleotides at both the 5' and 3' termini. These primary transcripts require subsequent processing at both ends to generate the mature species that are charged by their cognate tRNA aminoacyltransferases. The generally accepted model for tRNA processing involves endonucleolytic cleavages of primary transcripts by RNase E to generate pre-tRNAs (3-5), which contain a limited number of extra nucleotides at their 5' and 3' termini. Subsequently, the ribozyme RNase P endonucleolytically removes the extra nucleotides at the 5' terminus, while the 3' terminus is processed exonucleolytically by a combination of RNase T, RNase PH, RNase D, RNase BN, RNase II and PNPase (6,7).

Although RNase P has been shown to be involved in the maturation of 4.5S RNA (a component of the essential signal recognition particle) (8), other small RNAs (9), and in the processing of a few polycistronic mRNAs (10,11), its role in the endonucleolytic separation of polycistronic tRNA transcripts has only recently been demonstrated (12,13).

RNase P is universally conserved and has been found in all three domains of life (14-17) with the exception of the archaeon *Nanoarchaeum equitans* (17). Although RNase P is typically a ribonucleoprotein comprised of a conserved catalytic RNA subunit and a varying number of protein subunits (18,19), protein-only RNase P variants have been

recently described (20-24). *E. coli* ribonuclease P is an essential enzyme consisting of the C5 protein (encoded by *rnpA*) and the catalytic M1 RNA (encoded by *rnpB*) subunits (25,26). Under physiological conditions *in vitro*, the M1 RNA subunit has been shown to cleave pre-tRNA precursors on its own, albeit at a very slow rate (27). Addition of the C5 protein subunit increased the efficiency of the cleavage reactions dramatically (28). *In vivo*, the C5 subunit is essential for RNase P activity and cell viability (29).

The removal of the extra nucleotides at the 5' end of tRNAs by RNase P has been described as an essential step in the biogenesis of mature tRNA species for aminoacylation (30). With the discovery that RNase P separated the *valV valW* and *leuQ leuP leuV* primary transcripts (12), it was hypothesized that the essential function of RNase P might be related to the absence of a particular tRNA that was dependent on the enzyme for its initial processing, rather than its assumed importance in generating mature 5' termini (12). In fact, Kim *et al.* (31) suggested that they could complement a temperature sensitive allele of RNase P (*rnpA49*) by expressing $tRNA^{arg(CCG)}$ on a multicopy plasmid.

Based on these observations, we examined all 86 tRNA genes in *E. coli in silico* to determine if perhaps all of the tRNA genes for one specific amino acid were initially processed by RNase P, thereby generating a situation in which there would be a dramatic reduction in the level of a particular mature tRNA in the absence of RNase P. In such a scenario, loss of cell viability would result from the shortage of tRNAs charged with a specific amino acid rather than a general block to tRNA charging. Based on previous studies (3,7,12,13), many of the tRNAs including those for His, Leu, Pro, Trp,

Arg, Asn, Met, Cys, Ala, Gln, Ser, Phe, Thr, Ile, and Tyr were ruled out. However, both Val and Lys were possibilities, since we have previously shown that the two of the seven valine tRNAs (*valV* and *valW*, isotype 2) require RNase P for their initial processing (12) and the processing of the six lysine tRNA genes had not been previously studied. In fact, one lysine tRNA was part of a polycistronic transcript including three valine tRNAs (*valU valX valY lysV*), while the other five lysine tRNA genes were clustered together with two valine tRNAs (*lysT valT lysW valZ lysY lysZ lysQ*). However, it was indicated that the *lysT* gene cluster was synthesized as four separate transcripts (32).

Here we show that the *lysT* promoter drives the synthesis of a large polycistronic transcript that includes all seven tRNAs. More importantly, all seven valine tRNAs require RNase P for their initial processing. Thus upon inactivation of RNase P, functional valine tRNAs can no longer be generated, either isotype 1 or 2, because of the inability to generate valine pre-tRNAs from their primary transcripts. However, exogenous expression of both valine tRNA isotypes processed independently of RNase P did not complement an *rnpA49* mutant at the non-permissive temperature. We also show that RNase PH plays a major role in the 3' end maturation of type I valine isotype tRNAs.

MATERIALS AND METHODS

Bacterial strains

All the *E. coli* strains used in this study were derivatives of MG1693 (*thyA715 rph-1*) and are listed in Table 2.1. MG1693 is a *thyA715* derivative of MG1655 that has a single base

pair deletion in the *rph* gene (*rph-1*) resulting in a frameshift mutation, leading to the complete loss of RNase PH activity (33). The *rne-1* and *rnpA49* mutations, encoding temperature-sensitive RNase E and RNase P enzymes, respectively, do not support cell viability at 44ºC and have been previously described (34,35). A P1 phage lysate grown on SK2525 (*rnpA49 rph-1*) was used to transduce both SK10153 (*thyA715*) and SK9797 (*rne-1 rnzΔ500 rph-1*) to generate SK10521 and SK10300, respectively. SK5166 (*rnpA49 rph-1*) was transduced with a P1 lysate grown on SK4455 (*rnc-14 rph-1*) to construct SK10525. A P1 lysate grown on SK3170 (*rnlA2 rph-1*) was used to transduce SK2534 (*rne-1 rnpA49 rph-1*) to construct SK10523.

Plasmid constructions

The plasmids pAAK11 (*valU*$^{+}$/ Cm^{R}), pAAK15 (*argX*$^{+}$ /Cm^{R}) and pAAK17 (*rnpB*$^{+}$ /Cm^{R}) all contain the p15A origin of DNA replication (15-20 copies/cell) and express either *valU, argX* or the M1 RNA (*rnpB*), respectively, under the control of the *lac* promoter. Plasmid pAAK13 (*valW*$^{+}$ /Sm^{R}) is a 6-8 copy plasmid with a pSC101 origin of replication expressing *valW* under the control of the *lac* promoter. A PCR (polymerase chain reaction) fragment containing a *lac* promoter followed by a gene of interest (either *valU, valW, argX or rnpB*) and a Rho-independent transcription terminator [derived from *leuU*, (13)] was generated employing an overlapping PCR technique using Phusion® High-Fidelity DNA Polymerase (NEB). The resulting PCR products were cloned into the *Bam*HI/*Hin*dIII sites of either pBMK11(36) to construct pAAK11 (*valU*$^{+}$/ Cm^{R}), pAAK15 (*argX*$^{+}$ /Cm^{R}) and pAAK17 (*rnpB*$^{+}$ /Cm^{R}) or pMS421 (37) to construct pAAK13 (*valW*$^{+}$ /Sm^{R}).

All the plasmid constructions were confirmed by DNA sequencing of the cloned fragments (Eurofins MWG Operon). In addition, northern analysis was used to confirm that the IPTG induction of plasmids pAAK11, pAAK13, pAAK15 and pAAK17 led to increased intracellular levels of $tRNA^{Val1}$, $tRNA^{Val2}$, $tRNA^{Arg}$, and M1 RNA, respectively. Plasmid transformations were carried out as described previously (38). The sequences of various oligonucleotides used in the study are available on request.

Growth of bacterial strains

Bacterial strains were typically grown with shaking in Luria broth (39) supplemented with thymine (50 μg/ml). When appropriate, the medium also contained tetracycline (20 μg/ml), kanamycin (25 μg/ml), chloramphenicol (20 μg/ml) or streptomycin (20 μg/ml). Culture growth was monitored using a Klett-Summerson Colorimeter (No.42 Green filter). For temperature sensitive mutant strains, cultures were initially grown at 30ºC until they reached 50 Klett units above background and then shifted to 44ºC unless noted otherwise. All the cultures were maintained in exponential growth by periodic dilutions with pre-warmed medium. To collect samples for RNA extraction, RNase P (*rnpA49* allele) and RNase E (*rne-1* allele) mutant strains were shifted to 44ºC for 1 hr and 2 hr, respectively. The wild-type control strains were also shifted to 44ºC for one hour.

Isolation of total RNA

Total RNA for steady-state analysis was extracted using the method described by Mohanty *et al.* (40), with the following modifications. Cell pellets from 3.5 ml of cells were resuspended in 510 μl of Lysis buffer. After lysis, 71 μl of 20 mM acetic acid were

added. Following resuspension in one ml of 2 M LiCl, each sample was centrifuged at 16,000 g for 10 min. The resulting pellets were washed with 500 µl of chilled 70% ethanol, followed by centrifugation at 10,000 g for 10 min. The supernatants were removed and the pellets were spun again at 10,000 g for 1 min to remove any residual ethanol.

Total RNA for half-life analyses was extracted using the RNA*snap*™ method (41). Rifampicin (500 µg/ml) and nalidixic acid (20 µg/ml) were added to the cell cultures at 50 Klett units above background and the sample for 0 min time-point were collected after 70 seconds (40). All RNA samples were quantified using a NanoDrop (model 2000c, Thermo Scientific) apparatus. In addition, 500 ng of each RNA sample was separated on a 1% agarose gel and visualized by ethidium bromide staining to check for RNA integrity and to ensure equal loading.

Northern analysis and determination of *in vivo* aminoacylation levels

Northern analysis was carried out as described by Mohanty and Kushner (12). Briefly, 12 µg of total RNA from each strain were separated on either 6 or 8% polyacrylamide gels containing 8.3 M urea in TBE buffer or 1.2% agarose gels and were subsequently transferred on to a positively charged nylon membrane (Nytran™SPC, Whatman™). ^{32}P-labeled DNA oligonucleotides were used as probes. The blots were scanned with a PhosphorImager (Storm™ 840, GE Healthcare). Each membrane was often probed with different probes after stripping as previously described (40). Band intensities were determined using ImageQuant TL 5.2 software (GE Healthcare). The percentage of tRNA aminoacylation level was determined as previously described (42,43).

RESULTS

Processing of the *valU* operon is dependent on RNase P

The *E. coli* genome contains seven valine tRNAs organized into three separate gene clusters (Figure 2.1). The initial processing of *valV valW* transcripts containing two copies of valine isotype 2, has been shown to be completely dependent on RNase P cleavages at the mature 5' termini of both tRNAs that separate the two species (12). Since the spacer region between *valV* and *valW* is only four nucleotides (UCCU), we examined the spacer regions associated with the remaining five valine tRNAs, which all encode isotype 1. In fact, the *valU* operon has a four nucleotide spacer (CUUC) between *valY* and *lysV*, while in the *lysT* gene cluster there is a two nucleotide spacer (CC) between *valT* and *lysW* and a three nucleotide spacer (CUC) between *valZ* and *lysY* (Figure1). These observations suggested that RNase P might be involved in the initial processing of additional valine tRNAs, since these short spacer regions are not canonical RNase E cleavage sites (44,45).

Accordingly, we initially examined the processing of the *valU* operon in the *rph-1*, *rne-1 rph-1*, *rnpA49 rph-1* and *rne-1 rnpA49 rph-1* isogenic strains by northern analysis using operon specific oligonucleotide probes (Figure 2.2A). Since all five isotype I valine tRNAs have identical sequences, we initially probed a northern blot with a probe (b, Figure 2.2A) that was complementary to the mature valine type I coding sequence. No significant differences in processing intermediates were observed between the *rph-1* and *rne-1 rph-1* strains (Figure 2.2B, lanes 1-2). The most prominent species observed was a

doublet in which one band was slightly larger than the size of the mature tRNA (M). In addition, three high molecular weight species (V, VI and VII) were weakly visible (Figure 2.2B, lanes 1, 2).

The intensity of species V, VI, and VII increased dramatically in the absence of RNase P (*rnpA49*) along with an almost 20-fold decrease in the amount of the mature species (M) (Figure 2.2B, lane 3). In the *rne-1 rnpA49 rph-1* triple mutant, the intensity of the species V and VIII increased slightly along with small decreases in the levels of species VI and VII (Figure 2.2B, lane 4), suggesting a very minor role for RNase E in the absence of RNase P. The level of mature species (M) remained unchanged compared to the *rnpA49 rph-1* double mutant. In fact, the processed fraction (PF) for the mature $tRNA^{Val1}$ decreased from 0.76 and 0.95 in the *rph-1* and *rne-1 rph-1* strains, respectively, to 0.04 in the *rnpA49 rph-1* and *rne-1 rnpA49 rph-1* strains (Figure 2.2B, lanes 1-4).

The composition of all the processing intermediates seen in Figure 2.2B was subsequently determined by probing the blot with oligonucleotides that were complementary to various regions of the operon (Figure 2.2A). For example, probe a (complementary to the upstream region of *valU*) hybridized to all the processing intermediates (species V-IX, XI) with the exception of species X (Figure 2.2C, lanes 5-8). In contrast, probe g (complementary to the Rho-independent transcription terminator, Figure2A) only hybridized to species V (Figure 2.2C, lanes 21-24) demonstrating that it was the full-length transcript. These results were consistent with the predicted size (~438 nt) of the full-length *valU* operon transcript.

Since species VI hybridized only to probe a (Figure 2.2C, lanes 5-8), but not to probe g (Figure 2.2C, lanes 21-24), it was identified as the complete transcript minus the Rho-independent transcription terminator. The identity of species V and VI was further confirmed using probes c, d and e (Figure 2.2C, lanes 9-20). In fact, the Rho-independent transcription terminator associated with species V was removed primarily by RNase P, since its level increased only marginally in the *rne-1 rnpA49 rph-1* triple mutant compared to the *rnpA49 rph-1* double mutant (Figure 2.2, lanes 3, 4, 23, and 24).

Probe e (complementary to the intergenic region between *valY* and *lysV)* did not hybridize to either $tRNA^{Val}$ or $tRNA^{Lys}$ alone or to species VII (Figure 2.2C, lanes 17-20). Probe d hybridized to both species VI and VII, but not to species VIII (Figure 2.2C, lanes 13-16). These results suggested that species VII arose from a cleavage between *valY* and *lysV* tRNAs, while species VIII arose from a cleavage downstream of *valX*. The multiple bands labeled species X were observed with probes b and e in the *rnpA49 rph-1* strain (Figure 2.2B, lanes 3, 19), but disappeared in the *rne-1 rnpA49 rph-1* strain (Figure 2.2B, lanes 4, 20). This observation suggested that this group of processing intermediates containing valine coding sequences arose from occasional RNase E cleavages at multiple sites in the *rnpA49 rph-1* strain.

Species XI was detected by probes b and a in the *rnpA49 rph-1* (Figure 2.2B, 2.2C, lanes 3, 7,) and disappeared in the *rne-1 rnpA49 rph-1* strain (Figure 2.2B, 2.2C, lanes 4, 8). However, this band was not detected with probe c (complementary to the intergenic region between *valU* and *valX*), even in the *rnpA49 rph-1* strain (Figure 2.2C, lanes 11), indicating that in the absence of RNase P, RNase E cleaved inefficiently somewhere in

the intergenic region between *valU* and *valX*. Thus, intermediate XI was identified as a fragment containing *valU* with the 5' leader sequence and perhaps some portion of the intergenic region at its 3' end.

The detection of bands I- IV with probes b, e, and g (Figure 2.2B, 2.2C, lanes 4, 20, 24) was initially surprising, since their sizes were much larger than the full-length *valU* polycistronic transcript. However, upon examining the sequences of the *valU* operon and *lysT* gene cluster (32), we realized that the intergenic region between *valY* and *lysV* (CUUC, Figure 2.1) was similar to the intergenic regions between *valT* and *lysW* (CC, Figure 2.1) and *valZ* and *lysY* (CUC, Figure 2.1). Since all the five valine tRNAs and the six lysine tRNAs present in the *valU* operon and *lysT* gene cluster are identical to each other, respectively (Figure 2.1), we hypothesized that probes b and e (Figure 2.2A) would likely hybridize to RNA fragments originating from the *valU* operon as well as the *lysT* gene cluster. Additionally, we observed that the Rho-independent transcription terminators downstream of the *lysV* and *lysQ* genes (Figure 2.1) were identical in sequence (data not shown). Thus the large species observed with probes b, e, and g (Figure 2.2B, 2.2C, lanes 4, 20, 24) probably arose from transcripts originating from the *lysT* gene cluster (see below).

RNase PH plays an integral role in the final 3' end maturation of the valine type 1 tRNAs

As shown in Figure 2.2B, lanes 1, 2**,** a 1-2 nt larger species than the mature valine tRNA (M) was observed in the *rph-1* and *rne-1 rph-1* strains. Interestingly, the slightly larger species disappeared in the two *rnpA49* strains (Figure 2.2B, lanes 3, 4). Since our

experiments were carried out in an RNase PH defective strain (MG1693), we compared these results with those obtained from an isogenic *rph*$^{+}$ derivative of MG1693. As shown in Figure 2.2D, only the mature valine tRNA was observed in the presence of RNase PH.

***The lysT* gene cluster is transcribed as a polycistronic transcript**

Based on our observations with probes b, e, and g (Figure 2.2B, 2.2C), we hypothesized that the larger species (I, II, III) were part of the *lysT* gene cluster, even though it is indicated in the EcoCyc database that the seven genes are part of four separate transcripts (32). If all seven tRNAs in the *lysT* gene cluster were synthesized as a polycistronic transcript, a full-length species of over 1100 nt would be predicted. This species should hybridize to probes complementary to the 5' leader region of *lysT* (probe 1, Figure 2.3A), the 3' terminator region downstream of *lysQ* (probe g, Figure 2.3A), and the mature *lys* tRNA (probe f, Figure 2.3A). Accordingly, we performed northern analysis on steady-state RNA isolated from *rph-1, rnpA49 rph-1, rne-1 rph-1,* and *rne-1 rpnA49 rph-1* strains. The blot was first probed with an oligonucleotide complementary to the mature $tRNA^{Lys}$ sequence (probe f, Figure 2.3A). As predicted a species greater than 1100 nt in length (species I) was observed in the *rnpA49 rph-1* and *rnpA49 rne-1 rph-1* strains (Figure 2.3B, lanes 2, 4) along with multiple lower molecular weight species (II, III, IV, V, VIA, VIIA, X, XII, XIA and M). However, only species I hybridized to both probe 1 (Figure 2.3A, 3C, lanes 6, 8) and probe g (Figs. 3A, 2C, lane, 24) demonstrating that species I constituted the full-length *lysT* transcript. The larger species (I-IV) were barely visible in the *rph-1* and *rne-1 rph-1* strains (Figure 2.3B, lanes 1, 3).

Processing of the *lysT* polycistronic operon requires RNase P and is stimulated by RNase E

Unlike what was seen with the analysis of the *valU* operon, there was a single mature lysine tRNA species (M) in both the *rph-1* and *rne-1 rph-1* strains and surprisingly few other processing intermediates (Figure 2.3B, lanes 1, 3). In contrast, the amount of the mature $tRNA^{Lys}$ species (M) was significantly reduced in the *rnpA49 rph-1* and *rne-1 rnpA49 rph-1* mutants (Figure 2.3B, lanes 2, 4) with a concomitant increase in larger species (I-V, VIA, VIIA, X, XIA and XII). In fact, the processed fraction (PF) of mature $tRNA^{Lys}$ decreased from 0.95 and 0.89 in the *rph-1* and *rne-1 rph-1* strains to 0.17 and 0.02 in the *rnpA49 rph-1* and *rnpA49 rne-1 rph-1* mutants, respectively.

To ascertain the composition of the higher molecular weight species (I, II, III and IV), we probed the blot with oligonucleotides that were complementary to the intergenic regions between *lysW* and *valZ* (Figure 2.3A, probes 5, 6), *lysY* and *lysZ* (Figure 2.3A, probes 8, 9), and *lysZ* and *lysQ* (Figure 2.3A, probes 10, 11). With probes 10 and 11 (Figure 2.3C, lanes 25-28; data not shown), only species I was observed in both *rnpA49 rph-1* and *rne-1 rnpA49 rph-1* strains. However, with probes 8 and 9 (Figure 2.3C, lanes 17-20; data not shown), species I (full-length *lysT* operon) was observed in the *rnpA49 rph-1* strain, while species I and II were present in the *rne-1 rnpA49 rph-1* strain. These results suggested that species II was generated by a cleavage between the *lysZ* and *lysQ* tRNAs.

Since species II was only observed in the *rne-1 rnpA49 rph-1* triple mutant, it likely arose from RNase E cleavages of species I either prior the shift to the non-permissive temperature or due to residual RNase E activity associated with the *rne-1* allele.

Residual RNase E activity in *rne-1* strains has previously been reported (13). Similarly, species III was generated by a cleavage between *lysY* and *lysZ* tRNAs based on its hybridization to probes 5 and 6, but not the probes 8 and 9 (Figure 2.3C, lanes 13-20, data not shown). Species IV was most likely a *lysT valT lysW valZ* processing intermediate based on its molecular weight, but we could not unequivocally make this assignment.

Based on the data shown in Figure 2, the hybridization of probe f to species V in both the *rnpA49 rph-1* and *rnpA49 rne-1 rph-1* mutants (Figure 2.3B, lanes 2 and 4), represented the detection of the full-length *valU* polycistronic transcript (Figure 2.2B). Furthermore, the failure of probe 1 (Figure 2.3A) to hybridize to species V (Figure 2.3C, lanes 6 and 8) confirmed this identification. However, probe 1 hybridized to the two most prominent species of ~430 and ~140 nt (VIA and XII), respectively, in the *rnpA49 rph-1* double mutant (Figure 2.3C, lane 6). Since species VIA also hybridized to probes 2 and 4 (Figure 3A, 3C, lanes 10, 22), but not to probe 5 (Figure 2.3C, lane 14), this species was identified as *lysT valT lysW* processing intermediate that retained its 5' leader region.

Species XII was identified as a *lysT* intermediate with the 5' leader region, since it hybridized to probes f and 1(Figure3A, 3B, 3C, lanes 2, 6). The intensities of VIA and XII decreased significantly in the *rnpA49 rne-1 rph-1* triple mutant (Figure 2.3C, lane 8) along with concomitant increases in the intensities of species I-IV, suggesting these processing intermediates arose from RNase E cleavages within the larger *lysT* polycistronic transcript. It should be noted that species VIA (*lysT valT lysW*) is similar in size to species VI (Figure 2.2B, *valU valX valY lysV*).

Species VIIA was observed with probes that were complementary to the spacer region between *lysT* and *valT* (probes 2, 3) (Figure 2.3B, lanes 10, 12; data not shown), but was not detected with a probe complementary to the intergenic region between *valT* and *lysW* (probe 4) (Figure 2.3C, lanes 22, 24). Thus, species VIIA was a *lysT valT* processing intermediate that retained its 5' terminal sequence. It was designated as VIIA to distinguish it from species VII that was observed in Figure 2.2B, but was a *valU valX valY* processing intermediate and was almost identical in size. Species XIA was only observed with probe f (Figure 2.3B, lanes 2) and probe 9 (data not shown) suggesting it was a *lysZ* intermediate that retained some 3' sequences.

Species X (Figure 2.3B, lane 2) hybridized to probe 4 (complementary to the intergenic region between *valT* and *lysW*) (Figure 2.3C, lane 22) as well as probe 7 (data not shown). However, it failed to hybridize with probes 2, 5 and 8 (Figure 2.3C, lanes 10, 14 and 18). Thus, this species seemed to be a mixture of *valT lysW* and *valZ lysY* intermediates. The absence of species X in the *rne-1 rnpA49 rph-1* triple mutant indicated that these intermediates were generated by RNase E cleavages upstream of *valT*, *valZ* and *lysZ* in the absence of RNase P.

RNase P is the only enzyme that generates valine pre-tRNAs

In order to further confirm that RNase P was both essential and sufficient for the generation of the valine pre-tRNAs, we carried out half-life measurements on the three large *valU* operon transcripts (Figure 2, species V, VI and VII). We predicted that if other endoribonucleases were involved in processing this polycistronic transcript, even inefficiently, we would see the decay of one or more of these species in an *rnpA49 rph-1*

double mutant. In the *rph-1* control, both species V (the full-length transcript) and VII (*valU valX valY*) had identical half-lives of ~one minute (Figure 2.4, Table 2.2), while the half-life of species VI was estimated at <0.5 min (Figure 2.4, Table 2.2). In the *rne-1 rph-1* multiple mutant, all three species had half-lives of <0.5 min (Figure 2.4, Table 2.2). In contrast, in the absence of RNase P, the half-life species V increased over 7-fold, while that of species VI and VII increased over 30-fold (Figure 2.4, Table 2.2). In the absence of both RNase E and RNase P, all three species had half-lives of >30 min. These data provided further support that RNase E participated in the removal of the Rho-independent transcription terminator, but that this reaction only occurred slowly in the absence of RNase P.

Since the *lysT* polycistronic transcript was over 1100 nt in length, we carried out half-life experiments for this operon using 1.2% agarose gels. Unlike what was observed with the full-length *valU* transcript, we could not detect the full-length *lysT* transcript in either the *rph-1* or *rne-1 rph-1* strains even at time zero, indicating a half-life of <20 seconds (data not shown) in these genetic backgrounds. In contrast, the half-life of species I was >30 min in an *rnpA49 rne-1 rph-1* triple mutant (Table 2.2).

Although these data strongly suggested that RNase P was primarily responsible for the initial processing of all five valine type 1 tRNAs, we wanted to directly rule out the possibility that other known ribonuclease(s) besides RNase E might be involved in their processing. Accordingly, we tested a variety of multiple mutants that were deficient in RNase III (*rnpA49 rnc-14 rph-1*), RNase G (*rne-1 rnpA49 Δrng rph-1*), RNase LS (*rne-1 rnpA49 rnlA2 rph-1*), YbeY (*rnpA49 ΔybeY rph-1*), and RNase Z (*rne-1 rnpA49 Δrnz rph-1*)

as well as either RNase P or RNase E. None of the additional endonucleases tested affected the processing of the *valU* operon (data not shown). We also eliminated the possibility of residual RNase E activity in strains carrying the *rne-1* allele (13) by examining *valU* operon processing in an RNase E deletion strain (*rnpA49 Δrne-1018/ rng-219 rph-1*) (46) (data not shown).

Since species VII (Figure 2.2) was a *valU valX valY* species that was present in both the *rnpA49 rph-1* and *rnpA49 rne-1 rph-1* strains, we also tested the possibility that the *lysV* tRNA was removed by exonucleolytic processing from the 3' terminus. However, the processing of the *valU* primary transcript in multiple mutant strains deficient in PNPase (*pnp*) and RNase II (*rnb*) (*rne-1 pnp-7 rnb-500 rph-1*; *rnpA49 pnp-7 rnb-500 rph-1*; *rne-1 rnpA49 pnp-7 rph-1*; and *rne-1 rnpA49 rnb-500 rph-1*), was unchanged compared to a *rnpA49 rph-1* strain (data not shown).

However, these results, along with the data in Figure 2, did not provide a clear picture of the origin of species VII. Accordingly, we hypothesized that perhaps RNase P processing in an *rnpA49 rph-1* strain might be partially defective at permissive temperatures, leading to the generation of species VII prior to shift to 44°C. To test this hypothesis, we compared the processing of the *valU* operon in *rph-1*, *rne-1 rph-1,* and *rnpA49 rph-1* strains at 30°C, 37°C and 44°C. As shown in Figure 2.5, species VII was present in all three strains at 30°C, but there was significantly more in the *rnpA49* mutant. In fact, the processing of the *valU* primary transcript was almost as defective at 37°C in the *rnpA49 rph-1* strain as it was at 44°C.

Ectopic expression of mature valine tRNAs or tRNA$^{arg(CCG)}$ did not suppress the inviability associated with an *rnpA49* mutant

Since the loss of RNase P results in tRNAs with immature 5' ends, it has been assumed that such pre-tRNAs cannot be aminoacylated leading to an inhibition of protein synthesis and the subsequent loss of cell viability. However, recent studies suggest that tRNAs with immature 5' but mature 3' ends can be functional both *in vivo* and *in vitro* (13). On the other hand, the loss of RNase P activity could be lethal if all of a particular species of tRNA(s) remained unprocessed from their primary transcripts. From the analysis of the processing of the *valU* and *lysT* operons (Figs. 2, 3), it was evident that the amount of mature tRNA$^{Val(UAC)}$ was significantly reduced in the *rnpA49 rph-1* strains. Since the processing of tRNA$^{Val(GAC)}$ is also completely dependent on RNase P (12), we hypothesized that the loss of cell viability in the absence of RNase P arose from significantly reduced levels of functional valine tRNAs (See Figs. 2B, 3B, lanes 3 and 2, respectively). In order to test this hypothesis directly, we constructed a set of compatible plasmids from which the two valine isotypes, tRNA$^{val(GAC)}$ (*valW*) and tRNA$^{val(UAC)}$ (*valU*) were ectopically expressed in a way that did not require RNase P processing at either the 5' or 3' terminus. In both cases, the *valW* and *valU* genes were constructed such that transcription termination was controlled by the *leuU* Rho-independent transcription terminator, which is known to be removed by RNase E (13). Furthermore, to eliminate the need for RNase P processing of the tRNAs synthesized from the plasmids, they were designed such that transcription initiated at the mature 5' terminus of each tRNA.

To ensure that the tRNA species being expressed from the two plasmids were mature and functional, we measured the *in vivo* aminoacylation levels of the two valine tRNA isotypes. Surprisingly, the percentage of aminoacylation of both the valine tRNA isotypes was less than 40% in an *rph-1* strain (Table 2.3). This result was unexpected since other tRNAs have charging efficiencies ranging between 60-90% (47,48) in the same genetic background. As expected, however, the extent of aminoacylation of both valine tRNAs decreased between 1.9-2.3-fold (Table 2.3) in the absence of RNase P. The aminoacylation levels of both tRNAs returned to what was observed in the *rph-1* control upon ectopic expression of both tRNAs from plasmids in a *rnpA49 rph-1* strain (Table 2.3).

Subsequently, we examined the growth and cell viability of an *rnpA49 rph-1* strain in which both valine tRNAs were ectopically expressed. As a positive control we also overexpressed the M1 RNA component of RNase P, since increased levels of the M1 RNA have been shown to partially complement the *rnpA49* allele at 42°C (31,49). As shown in Figure 2.6, the simultaneous expression of $tRNA^{val(GAC)}$ and $tRNA^{val(UAC)}$ did not suppress the temperature sensitivity of the *rnpA49* strain. In contrast, overproduction of the *rnpB* gene did lead to a small improvement in the growth of the *rnpA49 rph-1* strain at 42°C (Figure 2.6A). In addition, there was no change in cell viability in the strain ectopically expressing both mature valine tRNAs (Figure 2.6B). It should be noted that identical results were obtained in an *rnpA49 rph*$^+$ strain (data not shown).

Ecotopic expression of the mature 4.5S RNA or tRNA$^{Arg(CCG)}$ does not suppress the inviability associated with the *rnpA49* allele

It has previously been suggest that overexpression of tRNA$^{Arg(CCG)}$ can complement an *rnpA49* mutation (31). However, as shown in Figure 2.6A, at 42^{o}C ectopic expression of this tRNA had no effect on the growth of an *rnpA49* mutant at the elevated temperature. Alternatively, it is known that RNase P is required to generate the mature 5' of the essential 4.5S RNA (25). In fact, it has been suggested that this processing reaction could be the essential function of RNase P in *E. coli* (15). Accordingly, we ectopically expressed a 4.5S RNA species that did not require RNase P processing at it 5' terminus. Increased levels of this species also did not suppress the conditional lethality of the *rnpA49* allele (data not shown).

DISCUSSION

Detailed northern analysis of both the *valU* and *lysT* operons (Figs. 2, 3) has demonstrated that all five of the valine isotype 1 pre-tRNAs are initially generated by RNase P cleavages. When coupled with our previous work showing that the two valine isotype 2 tRNAs (*valV* and *valW*) exclusively use RNase P for their initial processing (12), it is clear that all seven valine tRNA species in *E. coli* require RNase P for processing from their primary polycistronic transcripts. Surprisingly, however, the failure to mature the 5' termini only led to a ~ 2-fold reduction in the amount of aminoacylated valine tRNAs in an *rnpA49 rph-1* mutant (Table 2.3). Furthermore, the aminoacylated fraction of the valine tRNAs only marginally increased in a *rph*$^{+}$ strain (Table 2.3), even

though the level of the mature species increased significantly (Figure 2.2D). These results strongly suggest that extra nucleotides at the 5' terminus may not be a major *in vivo* impediment for many tRNA charging enzymes.

Of particular interest is the apparent mechanism by which RNase P processes both the *valU* and *lysT* polycistronic transcripts. In the analysis of the *valV valW* dicistronic transcript, it was suggested that RNase P cleavage at the 5' terminus of *valW* tRNA (Figure 2.1) might occur co-transcriptionally, since computer folding of the dicistronic transcript indicated that this RNase P site might be occluded (12). However, the data presented in Figs. 2 and 3 do not support such a mechanism for the *valU* and *lysT* polycistronic transcripts. Specifically, there are three possible mechanisms by which RNase P could process these transcripts: 1. Initiate cleavage at the 5' terminus of the first tRNA in the operon and proceed in the 5' → 3' direction to the end of the transcript; 2. Initiate processing by first removing the Rho-independent transcription terminator and proceeding in the 3'→ 5' direction to the proximal end of the transcript; or 3. Cleave the transcripts randomly with some sites being preferred over others.

The three possibilities can be distinguished by examining the processing intermediates obtained in the various *rnpA49* mutants (Figs. 2,3). For example, in the case of the *valU* operon, all of the primary processing intermediates observed after inactivation of RNase P retained their 5' termini (Figure 2.2B, 2C, lanes 3, 7). These data rule out RNase P initiating cleavage at the 5' proximal terminus and proceeding in the 5' → 3' direction. In addition, the failure to observe a significant number of processing intermediates lacking the 5' terminus also suggests that the RNase P cleavages are not

random, particularly since each of the intermediates detected in the *rnpA49 rph-1* mutant were shortened by one tRNA (Figure 2.2C, species V, VI, VII, VIII, and XI). It would thus appear that the *valU* operon is processed in the 3' → 5' direction with the first cleavage reaction being the removal of the Rho-independent transcription as outlined in Figure 2.7.

It should also be noted that the data shown in Figs. 2 and 4 demonstrate that the only role RNase E plays in the processing of the *valU* operon is to inefficiently remove the Rho-independent transcription terminator in the absence of RNase P, since the processing pattern of the full-length transcript is unchanged in the *rne-1 rph-1* mutant compared to the *rph-1* single mutant (Figure 2.2B, lanes 1, 2). Furthermore, the half-life data (Figure 2.4) and our analysis of other multiple mutants lacking RNase III, RNase G, YbeY, RNase Z and RNase LS (data not shown) demonstrated that RNase P was the only endonuclease that processed the full-length *valU* transcript. These results were very different from what was observed with the *metT* operon in which inactivation of RNase E by itself led to a significant change in the observed processing intermediates (40) and clearly demonstrate that RNase P is primarily responsible for the removal of the Rho-independent transcription terminator.

The processing of the *lysT* operon is somewhat more complex. RNase E does not play a significant role in the separation of any of the seven tRNAs, as judged by the lack of any new decay intermediates in the *rne-1 rph-1* strain (Figure 2.3B, lane 3). As seen with the *valU* operon the vast majority of the processing intermediates in both the *rnpA49 rph-1* and *rnpA49 rne-1 rph-1* strains retained their 5' termini, again suggesting

that RNase P initiates the processing of the large 1100 nt transcript at the 3' and proceeds in the 3'→ 5' direction. The increase in the amounts of species I-IV in the *rnpA49 rne-1 rph-1* triple mutant can be ascribed to the ability of RNase E to cleave within the large spacer regions between *lysT* and *valT*, *lysW* and *valZ*, *lysY* and *lysZ*, and *lysZ* and *lysQ* in the absence of RNase P. Importantly, it does not carry out these cleavages in the presence of RNase P (Figure 2.3B, lane 3). Thus we propose a processing pathway for the *lysT* operon (Figure 2.8) that is similar to that described for the *valU* operon.

Taken together, our data suggest that *in vivo* RNase P can efficiently process large transcripts starting from the 3' terminus. In fact, based on the half-life data (Table 2.2), the enzyme works more efficiently on the 1100 nt *lysT* transcript than the 440 nt *valU* transcript. In addition, although we have previously suggested that RNase P can remove a Rho-independent transcription terminator under certain circumstances (7), the data for the *valU* operon shows that it can carry out this reaction very efficiently (Figure 2.4). The fact that the enzyme appears to function with 3' → 5' directionally also indicates that there may not be preferred RNase P cleavage sites within the two polycistronic transcripts.

However, these models do not completely explain all of the data shown in Figs. 2, 3. For example, in the case of the *valU* operon the origin of species VII (*valU valX valY*) is still not clear. There is only a four nucleotide spacer between *valY* and *lysV* (CUUC). This sequence should not be cleaved by RNase E (44,45), even though there is an almost two-fold decrease in the amount of this species in the *rnpA49 rne-1 rph-1* triple mutant compared to the *rnpA49 rph-1* double mutant. When we examined the processing of the

valU operon in an *rnpA49 rne-1 Δrnz Δrng rnlA2* pentuple mutant, the decrease in species VII was identical to what was observed in the *rnpA49 rne-1* double mutant (data not shown). In addition, we could not demonstrate any exonucleolytic removal of *lysV* tRNA from the full-length transcript. Thus, one possibility for the origin of this species relates to the fact that RNase P activity in the *rnpA49* strain is already defective at both 30°C and 37°C, resulting in a significant amounts of this species being present at permissive growth temperatures (Figure 2.5). Alternative explanations include a possibility that the species arises from premature transcription termination at a site immediately downstream of *valY* or the existence of a yet unidentified endonuclease. It has generally been assumed that the essential function of RNase P is related to its role in generating the mature 5' termini on all 86 tRNA transcripts in *E. coli*, since extra nucleotides at the 5' terminus presumably would interfere with the binding of the tRNA to its appropriate charging enzyme. However, since inactivation of RNase P only led to 1-9-2.3-fold reductions in the fraction of the aminoacylated valine tRNAs (Table 2.3), it does not seem that this assumption is necessarily correct for all tRNAs in *E. coli*. These observations support our previous finding that a *leuU* tRNA with an extra nucleotide at the 5' terminus complemented the lethality associated with a *leuU* deletion mutant (13). Thus it may be that the presence of a limited number of extra nucleotides at the 5′ terminus is not a significant impediment to aminoacylation as long as the 3' end is mature.

Based on our determination of an over 30-fold reduction in the levels of type I valine tRNAs (Figure 2.2B) and similar findings for the type II valine tRNA (12), we

hypothesized that ectopic expression of both valine isotypes in a fashion that did not require RNase P to generate mature species might suppress the lethality associated with the *rnpA49* mutation. Much to our surprise, even though ectopic expression of the two valine tRNAs led to an increase in the amount of aminoacylated valine tRNAs (Table 2.3), no suppression of the growth defect was observed (Figure 2.6). In fact, when both valine tRNAs were simultaneously expressed, the strain grew more slowly than the *rnpA49* mutant on its own (Figure 2.6). The only improvement in growth after shift to 42°C occurred in the presence of increased expression of the *rnpB* gene, in agreement with previously reported results (49). Furthermore, we were not able to reproduce the work of Kim *et al.* (31), who suggested that overproduction of an arginine tRNA could suppress the *rnpA49* allele. In addition, we also ruled out the possibility that the processing of the 5' end of the 4.5S RNA was the essential function of RNase P (data not shown).

Several additional points are worth noting. In the Ecocyc database it has been reported that the *lysT* gene cluster is synthesized as four discrete transcripts (32). The data presented in Figs. 2 and 3 clearly slow that there is a single polycistronic transcript. Furthermore, the processing of the valine type 1 tRNAs was very dependent on RNase PH (Figure 2.2D) in agreement with the previous work of Li and Deutscher (6), in contrast to what was observed with the valine type 2 tRNAs (12). This result can be explained based on the unique specificity of RNase T (50). Specifically, the presence of C nucleotides immediately downstream of the CCA strongly inhibits RNase T activity (50). In the case of the type I valine tRNAs, all five species have at least one C immediately

downstream of the CCA (CCACUAC, CCACUUUC, CCACUUC, CCACC, CCACUC). In contrast, this is not the case with the *valV* and *valW* transcripts (CCAUCCU, CCAGAUU). It is also worth noting that even though the presence of RNase PH led to a single mature *val* tRNA species (Figure 2.2D), the fraction of aminoacylated type 1 tRNA did not significantly increase (Table 2.3). Thus, the relatively low levels of valine aminoacylation are not related to inefficient 3' end processing.

ACKNOWLEDGEMENTS

We thank Mark Stead, Valerie Maples and Khailee Marischuk for constructing some of the strains used in this study. This work was supported by NIH grants (GM57220 and GM81554 to S.R.K.)

TABLES AND FIGURES

Table 2.1: List of bacterial strains and plasmids used in this study

Strains	Genotype	Reference/ source
MG1693	*rph-1 thyA715*	*E. coli* Genetic Stock Center
SK2525	*rnpA49 rph-1 thyA715 rbsD296::*Tn*10* Tc^R	(4)
SK2534	*rne-1 rnpA49 rph-1 thyA715 rbsD296::*Tn*10* Tc^R	(4)
SK2549	*rne-1 rnpA49 rng::cat rph-1 thyA715 rbsD296::*Tn*10* Cm^R Tc^R	(51)
SK3170	*rnlA2::kan rph-1 thyA715* Km^R	Perwez & Kushner (unpublished results)
SK4455	*rnc-14 rph-1 thyA715* Tc^R	(52)

SK4484	*rne-1 rnpA49 rng::cat rnzΔ500::apr rnlA2::kan rph-1 rbsD296::Tn10* Cm^R Km^R Apr^R Tc^R	Maples and Kushner (unpublished results)
SK5166	*rnpA49 rph-1 thyA715 rbsD3163*::Tn*10* Km^R	Stead & Kushner (unpublished results)
SK5374	*ΔybeY::cat rnpA49 rph-1 thyA715 rbsD296::*Tn*10* Cm^R Tc^R	Stead & Kushner (unpublished results)
SK5665	*rne-1 rph-1 thyA715*	(53)
SK5704	*rne-1 pnp-7 rnb-500 rph-1 thyA715*	(53)
SK9797	*rne-1 rph-1 rnzΔ500::kan thyA715* Km^R	(54)
SK10153	*thyA715*	(43)
SK10300	*rne-1 rnpA49 rnzΔ500::kan rph-1 thyA715 rbsD296::*Tn*10* Tc^R Km^R	This study
SK10451	*rnpA49 pnp-7 rnb-500 rph-1 thyA715 rbsD296::*Tn*10* Tc^R	(12)
SK10460	*Δrne-1018::bla/ rng-219 rnpA49 rph-1 thyA715 rbsD296::*Tn*10* Sm^R/Sp^R Ap^R Km^R Tc^R	(46)
SK10523	*rne-1 rnpA49 rnlA2::kan rph-1 thyA715 rbsD296::*Tn*10* Tc^R Km^R	This study
SK10525	*rnc-14 rnpA49 rph-1 thyA715 rbsD296::*Tn*10* $Tc^R Km^R$	This study
SK10530	*rnpA49 rph-1 thyA715 rbsD296::*Tn*10* Tc^R/pAAK11	This study
SK10531	*rnpA49 thyA715 rbsD296::Tn10* Tc^R/pAAK11	This study
SK10533	*rnpA49 rph-1 thyA715 rbsD296::*Tn*10* Tc^R/pAAK13	This study
SK10534	*rnpA49 thyA715 rbsD296::Tn10* Tc^R/pAAK13	This study
SK10537	*rnpA49 rph-1 thyA715 rbsD296::*Tn*10* Tc^R /pAAK15	This study
SK10538	*rnpA49 thyA715 rbsD296::Tn10* Tc^R/pAAK15	This study
SK10539	*rnpA49 rph-1 thyA715 rbsD296::*Tn*10* Tc^R/pAAK17	This study
SK10540	*rnpA49 thyA715 rbsD296::*Tn*10* Tc^R/pAAK17	This study
SK10541	*rnpA49 rph-1 thyA715 rbsD296::*Tn*10* Tc^R/pAAK11 pAAK13	This study
SK10542	*rnpA49 thyA715 rbsD296::*Tn*10* Tc^R/pAAK11 pAAK13	This study
Plasmids		

pMS421	*lacI*$^{q+}$, Sm^{R}	(37)
pBMK11	*pcnB*$^{+}$, Cm^{R}	(36)
pAAK11	pBMK11, *valU*$^{+}$,Cm^{R}	This study
pAAK13	pBM23, *valW*$^{+}$,Sm^{R}	This study
pAAK15	pBMK11, *argX*$^{+}$,Cm^{R}	This study
pAAK17	pBMK11, *rnpB*$^{+}$,Cm^{R}	This study

Table 2.2: Half-lives of *valU* and *lysT* operon transcripts in various strains.

Species designations are as indicated in Figs. 2 and 3. Half-lives were determined as described in the Materials and Methods. Data reflect the averages of at least three independent determinations.

(A) *valU*

Species	Half-life (min)			
	rph-1	***rnpA49 rph-1***	***rne-1 rph-1***	***rnpA49 rne-1 rph-1***
V	1.0 ± 0.4	7.7 ± 1.4	<0.5	>30
VI	<0.5	>30	<0.5	>30
VII	1.1 ± 0.1	>30	<0.5	>30

(B) *lysT*

Species	Half-life (min)			
	rph-1	***rnpA49 rne-1 rph-1***	***rnpA49 rneΔ1019 rng-219 rph-1***	***rnpA49 rne-1 Δrng rnlA2 Δrnz rph-1***
I	<0.3	>30	>30	>30

Table 2.3: Aminoacylation levels of mature valine tRNAs.

The percentage of each aminoacylated tRNA was determined by the method of Varshney (42) as previously described (47). Data shown is the average of at least two independent determinations.

tRNA isotype/genes	**Percentage of aminoacylated tRNA**			
	rph-1	***rnpA49 rph-1***	***rnpA49 rph-1/ valU⁺ valW⁺***	***rph⁺***
Val1/*valU, X,Y, T, Z*	39 ± 2	17 ± 6	37 ± 3	41 ± 5
Val2/*valV, W*	38 ± 4	20 ± 3	37 ± 3	47 ± 5

Figure 2.1: Schematic diagram of the seven valine tRNAs in *E. coli* (not drawn to scale): *valV valW*; *valU valX valY lysV;* and *lysT valT lysW valZ lysY lysZ lysQ* operons. The length of the intergenic and the nucleotide sequences of the short spacers are indicated. *valV* and *valW* encode for $tRNA^{val(GAC)}$ and are similar in sequence. *valU*, *valX*, *valY*, *valT* and *valZ* encode $tRNA^{val(UAC)}$ and are identical in sequence. The valine and lysine tRNAs with identical nucleotide sequences are similarly colored.

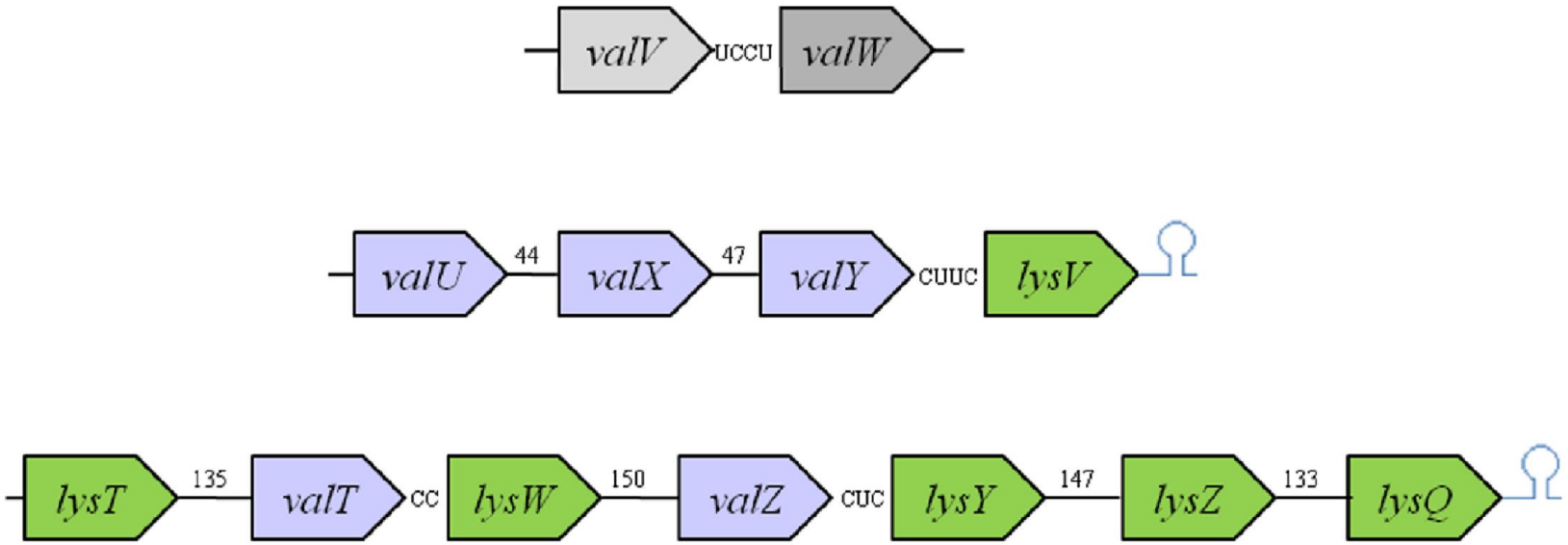

valV
UCCU
valW
valU
44
valX
47
valY
CUUC
lysV
lysT
135
valT
CC
lysW
150
valZ
CUC
lysY
147
lysZ
133
lysQ

Figure 2.2: Analysis of *valU* operon processing. (A) Schematic diagram of the *valU* operon (not drawn to scale). Relative positions of the oligonucleotide probes (a: valU-UP, b: valUXYTZ, c: valU-X, d: valX-Y, e: valY-lysV, f: lysmature, g:lysV-TER) used in northern analyses are shown. (B, C) Northern analysis of *valU* operon. The specific oligonucleotide probe used for each blot is indicated in the bottom left corner of the blot. The genotypes of the strains used are indicated on the top of each autoradiogram. All the strains are isogenic derivatives of MG1693 (*rph-1*). The RNA molecular size standards (nucleotides) (Fermentas) are shown to the left of the first image. The numerical designations of the different species along with their graphical structures are shown to the right of the first blot (B). The lane numbers are indicated at the bottom of the autoradiograms. Relative quantity (RQ) of mature $tRNA^{Val}$ (M) in various genetic backgrounds was calculated by setting a level of one in the *rph-1* strain. Processed fraction (PF) represents the fraction of mature tRNA relative to the total amount of processed and unprocessed species of that tRNA. Each value represents the average of at least three independent determinations. (D) Analysis of the role of RNase PH in the final maturation of the five valine type I tRNAs.

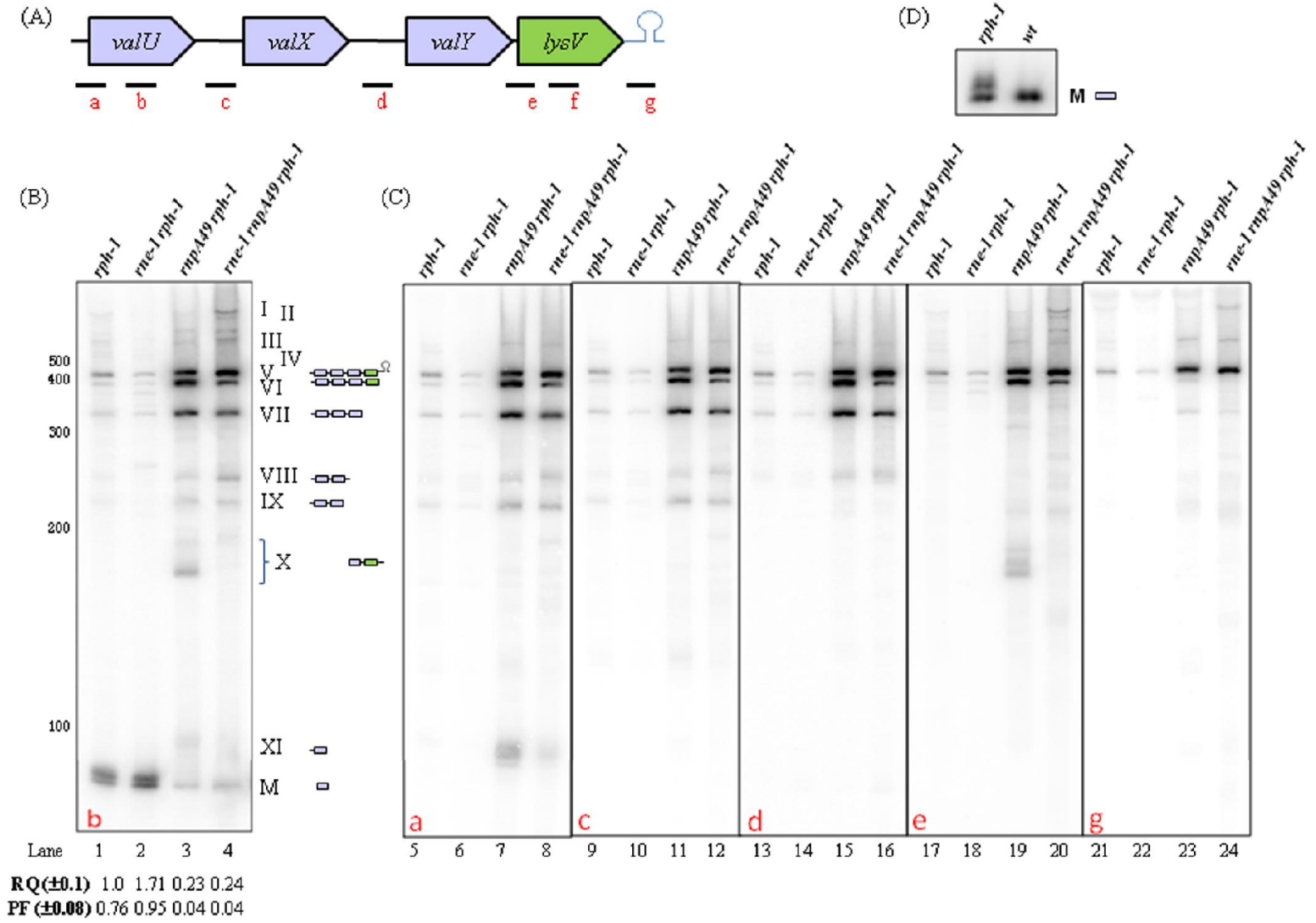

(A)
valU
valX
valY
lysV
a
b
c
d
e
f
g
(D)
rph-1
wt
M
(B)
rph-1
rne-1 rph-1
rnpA49 rph-1
rne-1 rnpA49 rph-1
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
M
500
400
300
200
100
b
Lane 1 2 3 4
RQ(±0.1) 1.0 1.71 0.23 0.24
PF (±0.08) 0.76 0.95 0.04 0.04
(C)
a
c
d
e
g
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

Figure 2.3: Analysis of the *lysT* operon processing. (A) Schematic diagram of the operon (not drawn to scale). Relative positions of the oligonucleotide probes (1: lysT-UP, f: lysmature, 2: lysT-valT1, 3: lysT-valT3, b: valUXYTZ, 4: valT-lysW, 5: lysW-valZ1, 6: valZ-UP2, 7: valZ-lysY, 8: lysY-lysZ1, 9: lysY-lysZ2, 10: lysZ-lysQ1, 11: lysZ-lysQ2, g: lysV-TER). (B, C) Northern analysis of *lysT* operon. The specific oligonucleotide probe used in each blot is indicated in the bottom left corner of the blot. The genotypes of the strains used are indicated on the top of the each autoradiogram. All the strains are isogenic derivatives of MG1693 (*rph-1*). The RNA molecular size standards (nucleotides) (Fermentas) are shown to the left of the first blot. The numerical designations of the different species along with their graphical structures are shown to the right of the blot. The lane numbers are indicated at the bottom of the autoradiograms. Two independent northerns were run (one was probed with f, 1, 2, 5 and 8, second was probed with 4 and 10). The RQ and PF values were calculated as described in the legend to Figure 2.2.

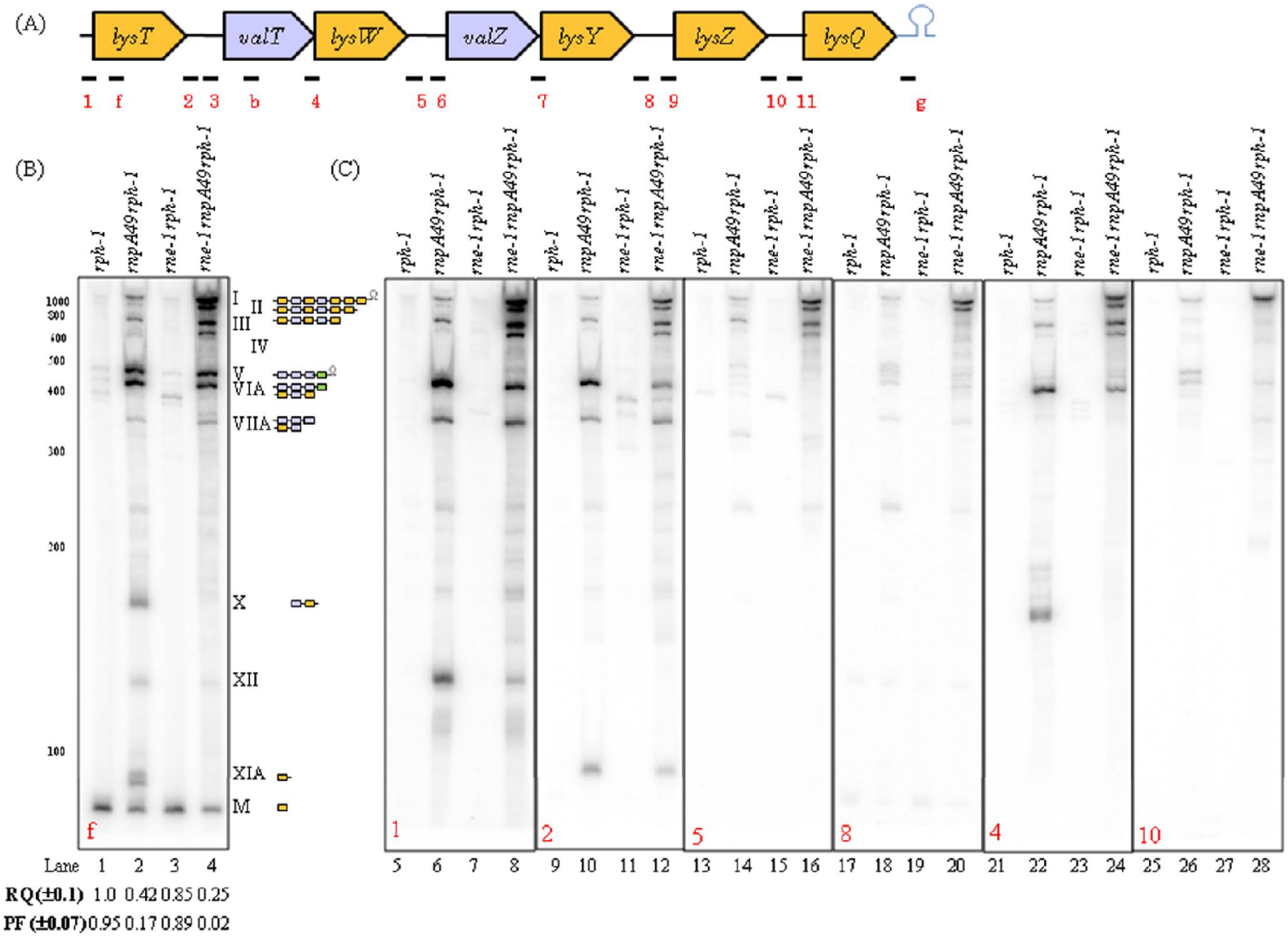

(A)
lysT
valT
lysW
valZ
lysY
lysZ
lysQ
1 f 2 3 b 4 5 6 7 8 9 10 11 g
(B)
(C)
rph-1
rnpA49rph-1
rne-1 rph-1
rne-1 rnpA49rph-1
1000
900
600
500
400
300
200
100
I
II
III
IV
V
VIA
VIIA
X
XII
XIA
M
f
1
2
5
8
4
10
Lane 1 2 3 4
RQ(±0.1) 1.0 0.42 0.85 0.25
PF (±0.07) 0.95 0.17 0.89 0.02
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28

Figure 2.4: Northern analysis of processing intermediates of *valU* operon. (A) Northern analysis of the decay of *valU* operon. The genotypes of the strains and time-points are indicated on the top of the autoradiograms. The blots were probed with probe b (Figure 2.2A). The numerical designations of the different species with their graphical structures are shown to the right and are identical to those shown in Figure 2.2.

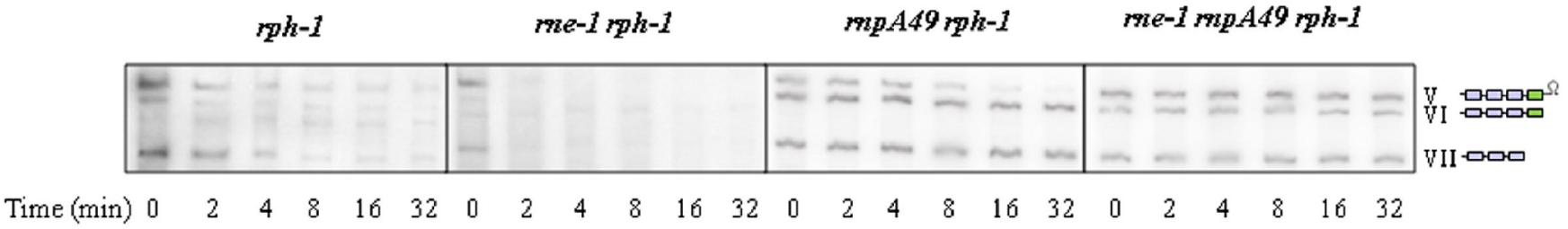

rph-1
rne-1 rph-1
rnpA49 rph-1
rne-1 rnpA49 rph-1
V
VI
VII
Ω
Time (min) 0 2 4 8 16 32 0 2 4 8 16 32 0 2 4 8 16 32 0 2 4 8 16 32

Figure 2.5: RNase P processing in an *rnpA49* mutant is defective at permissive temperatures. Northern analysis of total steady-state RNA was performed as described in the Materials and Methods section. For the 30°C and 37°C samples, the strains were grown at that particular temperature. For the 44°C samples, the strains were initially grown at 30°C and then shifted to 44°C for 60 min. The genotypes of the strains and the temperatures are indicated at the top of the autoradiogram. The RNA molecular size standards (nucleotides) (Fermentas) are shown to the left of the blot. The designations of the different species and their representative structures are shown to the right. The lane numbers are indicated at the bottom of the autoradiogram. The blot was probed with probe a (Figure 2.2A).

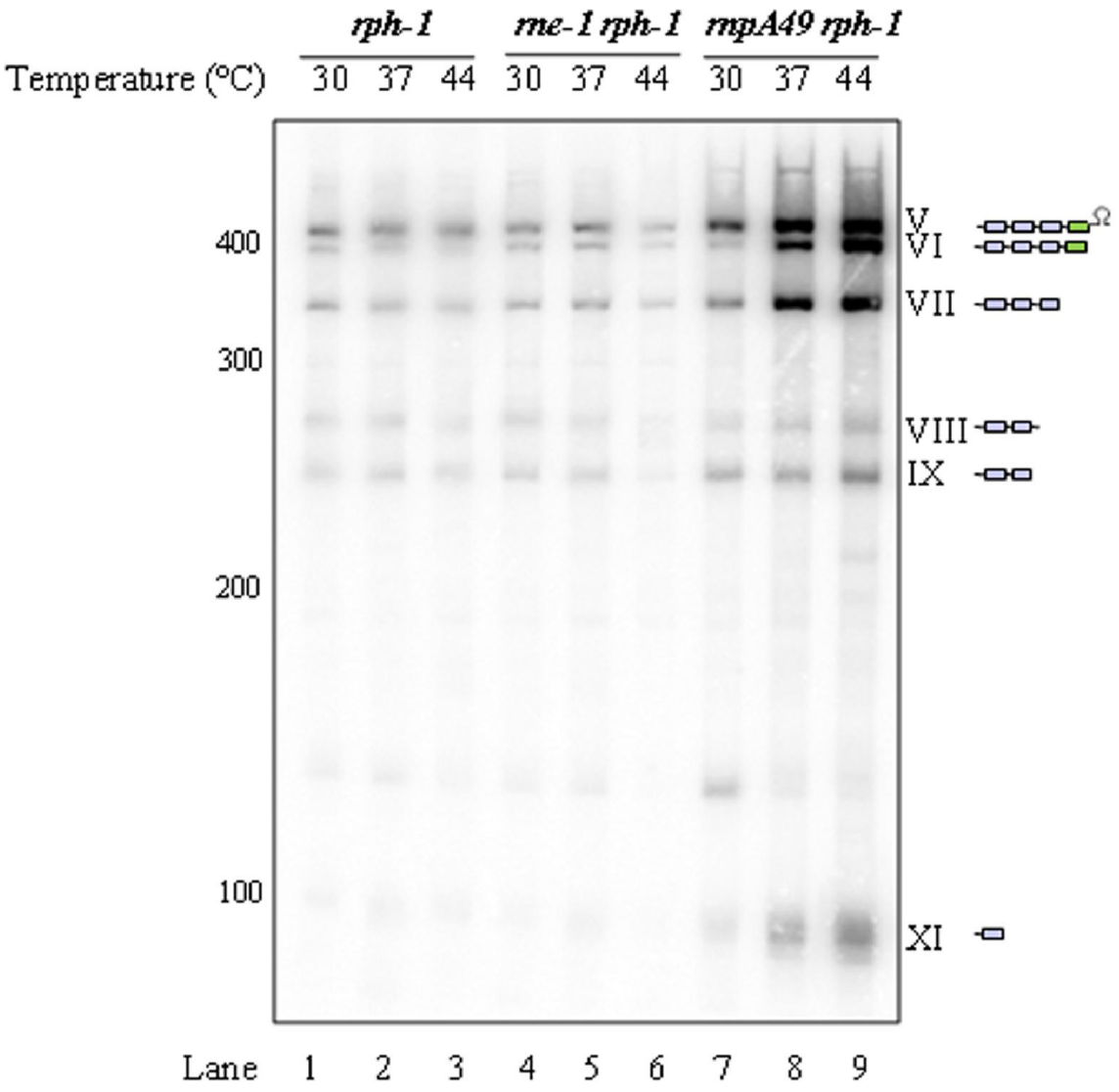

rph-1
rne-1 rph-1
rnpA49 rph-1
Temperature (°C)
30 37 44 30 37 44 30 37 44
400
300
200
100
V
VI
VII
VIII
IX
XI
Ω
Lane 1 2 3 4 5 6 7 8 9

Figure 2.6: Growth curve (A) and cell viability (B) analysis of various strains. All strains were initially grown at 30 °C until 50 Klett units above background when IPTG was added to a final concentration of 350 μM. For this experiment the cultures were shifted to 42°C, since it has been previously shown that overproduction of *rnpB* does not complement at 44°C (49). The cultures were diluted with pre-warmed LB/thymine or LB/thymine/IPTG to maintain them in exponential growth. The optical densities (OD) were measured with a Klett-Summerson Colorimeter (No. 42 Green filter). The viable cell counts (CFU) were determined starting from the temperature shift (0 min) by plating on LB/thymine agar plates at 30°C. x: MG1693 (*rph-1*); ♦: SK2525 (*rnpA49 rph-1*); ▲: SK10537 (*rnpA49 rph-1 argX*$^+$);: ●: SK10541 (*rnpA49 rph-1 valU*$^+$ *valW*$^+$); ■: SK10539 (*rnpA49 rph-1 rnpB*$^+$).

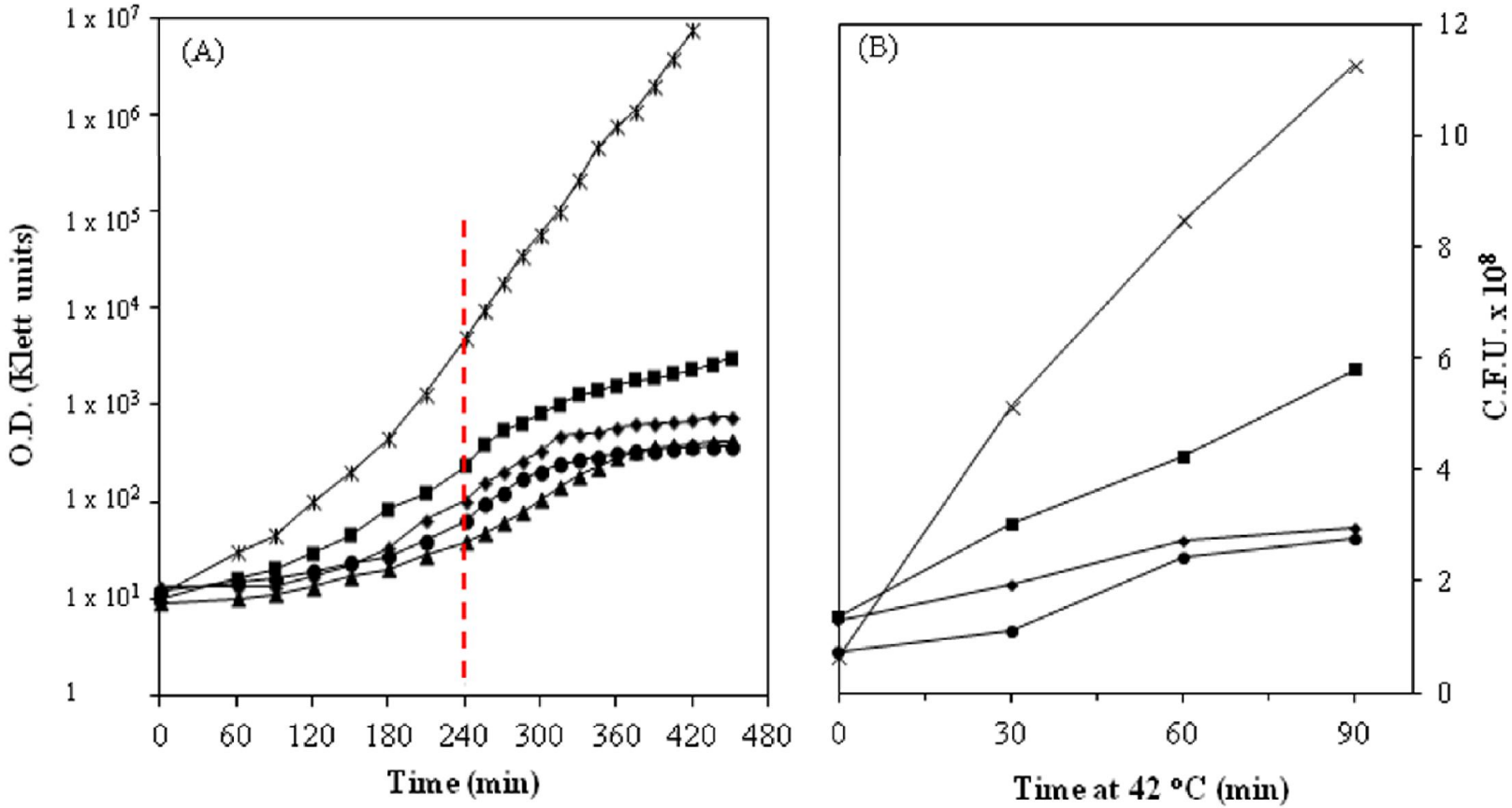

(A)
O.D. (Klett units)
1 x 10^7
1 x 10^6
1 x 10^5
1 x 10^4
1 x 10^3
1 x 10^2
1 x 10^1
1
0
60
120
180
240
300
360
420
480
Time (min)
(B)
C.F.U. x 10^8
12
10
8
6
4
2
0
0
30
60
90
Time at 42 °C (min)

Figure 2.7: Processing pathway for the *valU* polycistronic transcript. Similar to what has been observed with the *valV valW* and *secG leuU* primary transcripts (12,13), RNase P (upward black arrows) is the primary processing enzyme for the *valU* transcript. In addition, RNase P efficiently removes the Rho-independent transcription terminator (upward black arrow). Subsequent sequential cleavages occur in the 3' → 5' direction at the mature 5' termini of *lysV*, *valY*, *val X* and *valU* (upward black arrows). The *valU* and *valX* pre-tRNAs will have long 3' termini of 44 and 47 nt, respectively (Figure 2.1) that will be initially processed by a combination of RNase II and PNPase (12,55). Subsequently, the final processing of the valine pre-tRNAs will be carried out primarily by RNase PH because of the presence of C residues downstream of the CCA determinant (50). The final processing of the *lysV* pre-tRNA employs RNase T. In the absence of RNase P, RNase E inefficiently removes (dashed arrow) the Rho-independent transcription terminator. Transcripts are not drawn to scale.

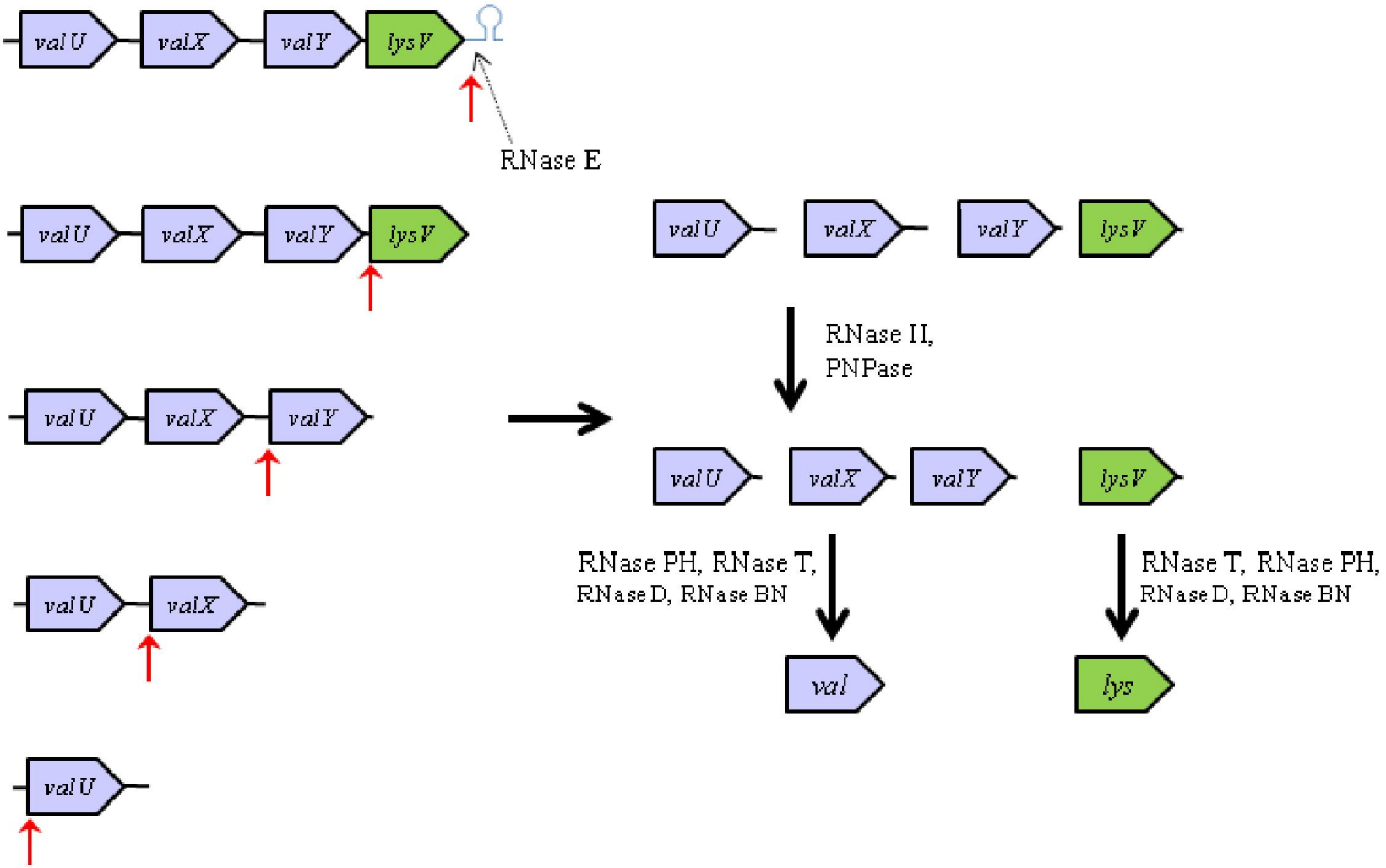
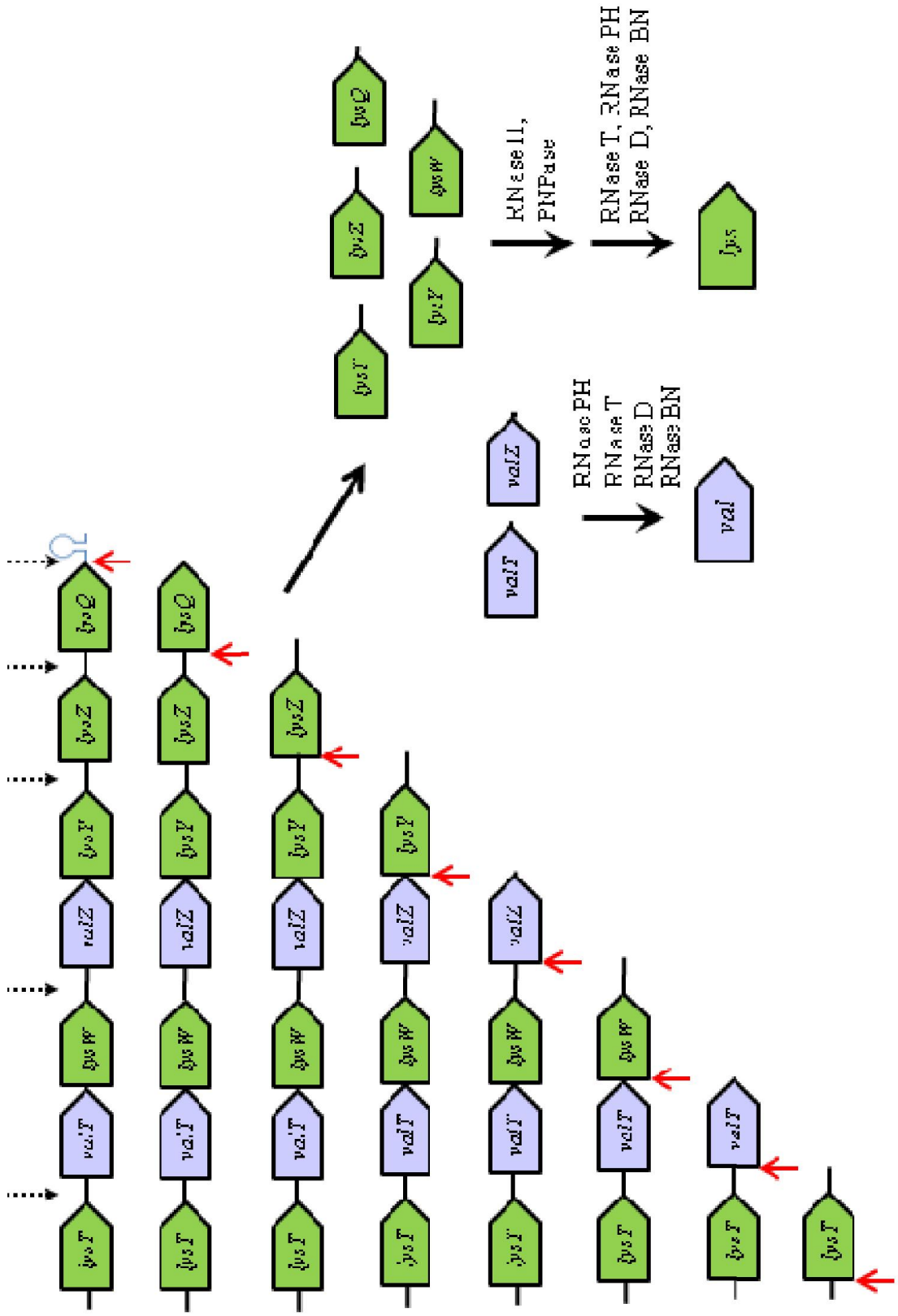

valU
valX
valY
lysV
RNase E
RNase II,
PNPase
RNase PH, RNase T,
RNase D, RNase BN
RNase T, RNase PH,
RNase D, RNase BN
val
lys

Figure 2.8: Processing pathway for the *lysT* polycistronic operon. Similar to what was described in Figure 2.7, after RNase P removes the Rho-independent transcription terminator (upward black arrow), subsequent sequential cleavages will occur in the 3' → 5' direction at the mature 5' termini of *lysQ*, *lysZ*, *lysZ*, *lysW*, *valT*, and *lysT* (upward black arrows) to generate pre-tRNAs containing between 2-150 nt extra nucleotides at their 3' ends (Figure 2.1). The long 3' ends of the five *lys* pre-tRNAs will be initially processed by a combination of RNase II and PNPase (12,55). Final maturation of the *lys* tRNAs primarily is carried out by RNase T, while the final processing of the two valine species is done by RNase PH. In the absence of RNase P, RNase E (dashed arrows) can cleave within the long spacer regions between *lysT* and *valT*, *lysW* and *valZ*, *lysY* and *lysZ, and lysZ* and *lysQ.* However, RNase E does not separate *valT* and *lysW* or *valZ* and *lysY*. Transcripts are not drawn to scale.


lysT
valT
lysW
valZ
lysY
lysZ
lysQ
RNase II,
PNPase
RNase T, RNase PH
RNase D, RNase BN
lys
RNase PH
RNase T
RNase D
RNase BN
val

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CHAPTER 3

LOSS OF POLYADENYLATION IN *ESCHERICHIA COLI* SUPPRESSES THE TEMPERATURE SENSITIVITY OF AN RNASE P MUTANT[1]

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INTRODUCTION

In *Escherichia coli*, all the transfer RNA (tRNA) genes are initially transcribed as precursors with additional sequences at both the 5′ and 3′ termini. These precursors undergo processing by exo- and endo-nucleolytic ribonucleases to remove the extra nucleotides and generate the mature tRNA sequence. Briefly, Ribonuclease P (RNase P) by itself or in combination with RNase E generates the mature 5′ termini while the 3′ termini are generated by RNase T, RNase PH, RNase D and RNase BN (aka RNase Z) (1-6). Polynucleotide phosphorylase (PNPase) and RNase II may also be involved in the 3′ end processing.

RNase P is a ubiquitous and essential enzyme found in all three domains of life, with *Nanoarchaeum equitans*, an obligatory parasitic archaeon, being the only known exception (7-10). Although it is typically a ribozyme (11,12), protein-only variants of RNase P have been described recently (13-17). In *E. coli*, it is comprised of the C5 protein (encoded by *rnpA* gene) and the catalytic M1 RNA (encoded by *rnpB* gene) (18). A majority of the genetic studies to understand the biological functions of RNase P, including its role in 5′ end processing of tRNA precursors, have been conducted using temperature sensitive mutants of either the protein or the RNA subunit (19,20).

Recently it has been demonstrated that tRNAs as well as other stable RNAs are substrates for polyadenylation by poly(A) polymerase I (PAP I, encoded by *pcnB* gene), the primary enzyme responsible for polyadenylation in *E. coli* (21). While few, if any, tRNAs with poly(A) tails are found in wild-type cells, any defects in 3′ end processing

result in accumulation of tRNA precursors with poly(A) tails. Further, PAP I was shown to naturally compete with RNase T and RNase PH (the primary exonucleases responsible for tRNA processing) for the 3′ ends of tRNAs. In wild-type cells, accurate and efficient processing of the 3′ ends of tRNA precursors generates mature tRNAs which are quickly charged with their cognate amino acid, leaving little opportunity for PAP I to add 3′ tails to the tRNAs. When 3′ end processing is compromised due to absence of RNase T and/or RNase PH (21,22), majority of the tRNA precursors become substrates for polyadenylation by PAP I. Consequently, the aminoacylation level of tRNAs is drastically reduced, while the cellular growth rate increases comparably. This competition between PAP I-mediated polyadenylation and RNase T/RNase PH mediated exonucleolytic activities has been suggested to constitute a mechanism to regulate the level of functional tRNAs in the cell.

While the 3′ ends of tRNAs must be completely and accurately processed to the CCA determinant for aminoacylation (23), there is flexibility at the 5′ end. The ability of a mutant $tRNA^{Leu}$ carrying an extra nucleotide (G_{-1}) to complement a *leuU* deletion mutant in *E. coli* suggests that tRNAs with immature 5′ termini can be aminoacylated (4). Indeed, $tRNA^{His}$ contains an additional G_{-1} residue nearly universally, which is essential for efficient aminoacylation by histidinyl-tRNA synthetase (HisRS) (24,25). Similar examples of naturally occurring tRNAs containing one additional nucleotide at the 5′ termini, including mt-$tRNA^{Phe}$, $tRNA^{Tyr}$ and $tRNA_i^{Met}$, which are efficient substrates for their cognate aaRS have been previously described (10,26,27). However, the fate of tRNA

precursors with longer 5′ leaders in the absence of RNase P has not been studied. We were curious to investigate if these tRNA precursors were substrates for polyadenylation or if they could be charged by their cognate aaRS enzymes despite the presence of extra sequences at the 5′ end.

In this report, we show that the loss of polyadenylation activity in *E. coli*, due to disruption of the *pcnB* gene, leads to suppression of the temperature sensitivity of an RNase P mutant. Loss of polyadenylation also results in an increase in the cellular level of RNase T, suggesting that poly(A) polymerase I plays a role in regulating the *rnt* message . Further, we show that the presence of short 5′ leaders (1-5) does not inhibit the aaRS enzymes, and pre-tRNAs with such extra sequences are aminoacylated efficiently. In contrast, for pre-tRNAs with longer 5′ leaders in the absence of RNase P, the aminoacylation levels are reduced dramatically.

MATERIALS AND METHODS

Bacterial strains and plasmids

All the strains used in this study were derived from MG1693 (*thyA715 rph-1*) and are listed in Table 1. MG1693 has a single base pair deletion in the *rph* gene, resulting in a frame-shift mutation, which causes the complete loss of RNase PH activity. The *rnpA49* allele encodes a temperature-sensitive RNase P enzyme that does not support cell viability at 44 ºC and has been previously described (28).

SK10153 was transduced with a P1 lysate grown on SK2525 (*rnpA49 rph-1*) to generate SK10521 (*rnpA49*). A P1 lysate grown on SK7988 (*ΔpcnB rph-1*) was used to transduce SK2525 and SK10521 to generate SK10297 (*rnpA49 ΔpcnB rph-1*) and SK10522 (*rnpA49 ΔpcnB*) respectively. A P1 lysate grown on SK10148 (*Δrnt rph-1*) was used to transduce SK2525 to generate SK10700 (*rnpA49 Δrnt rph-1*).

Plasmid pAAK17 (*rnpB*$^{+}$ /Cm^{R}) contains the p15A origin of DNA replication (15-20 copies/cell) and expresses the M1 RNA (*rnpB*) under the control of the *lac* promoter. A PCR fragment containing a *lac* promoter, the *rnpB* gene and a Rho-independent transcription terminator [derived from *leuU* gene (5)] was generated by overlapping PCR technique using Phusion® High-Fidelity DNA Polymerase (NEB). The resulting PCR product was cloned into the *Bam*HI/*Hin*dIII sites of pBMK11 (29) to construct pAAK17 (*rnpB*$^{+}$ /Cm^{R}). Northern analysis was used to confirm that IPTG induction led to increased intracellular level M1 RNA. Plasmid transformations were carried out as described previously (30).

Growth of bacterial strains

Bacterial strains were typically grown with shaking in Luria broth supplemented with thymine (50 μg/ml). When appropriate, tetracycline (20 μg/ml), kanamycin (25 μg/ml) or chloramphenicol (20 μg/ml) were added to the culture medium. Cellular growth was monitored using a Klett-Summerson Colorimeter (No.42 Green filter). All the cultures were initially grown at 30 °C until they reached 50 Klett units above background and then shifted to 44 °C, unless mentioned otherwise. For strains containing pAAK17, IPTG

(350 μM) was added 30 min before temperature shift. All the cultures were maintained in exponential growth by periodic dilutions with pre-warmed LB/thymine or LB/thymine/IPTG.

Isolation of total RNA, northern analysis, poly(A) sizing assay, RT-PCR cloning, sequencing of 5′-3′ ligated transcripts and determination of *in vivo* aminoacylation level

To collect samples for RNA extraction, all the strains were shifted to 44 °C for 1 hr. Total RNA for steady-state analysis was extracted using the method described by Mohanty *et al.* (31), with the following modifications. Cell pellets from 3.5 ml of cells were resuspended in 510 μl of Lysis buffer. After lysis, 71 μl of 20 mM acetic acid were added. Following resuspension in one ml of 2 M LiCl, each sample was centrifuged at 16,000 g for 10 min. The resulting pellets were washed with 500 μl of chilled 70% ethanol, followed by centrifugation at 10,000 g for 10 min. The supernatants were removed and the pellets were spun again at 10,000 g for 1 min to remove any residual ethanol.

The details of northern analysis, poly(A) sizing assay, identification of the 5′ and 3′ ends of tRNAs and *in vivo* determination of aminoacylation level of tRNAs have also been described previously (21,31). The sequences of various oligonucleotides used in the study are available on request.

Western analysis

Cellular levels of RNase T and RNase PH were determined as described (21,22). All the strains were grown initially at 30 °C and shifted to 44 °C for 1 hr after they reached a cell density of 50 Klett units.

RESULTS

Loss of polyadenylation suppresses the *rnpA49* mutant allele

In a recent report, Mohanty *et al*. demonstrated that inactivation of PAP I in the *rnt rph-1* mutant resulted in faster generation time, presumably because of more efficient processing of the 3′ ends of tRNA precursors (21). We were curious to test if the disruption of PAP I in an RNase P mutant would result in a similar improvement of growth. As shown in Figure 3.1, indeed the *rnpA49 ΔpcnB rph-1* mutant grew better than the *rnpA49 rph-1* mutant. In fact, while the *rnpA49 rph-1* mutant stopped growing after ~2 hr at the non-permissive temperature of 42 °C, the *rnpA49 ΔpcnB rph-1* strain kept growing, suggesting that the absence of PAP I was able to suppress the temperature sensitivity of the *rnpA49* allele. It has been previously established that over-expression of the catalytic M1 RNA component of RNase P holoenzyme can partially suppress the temperature sensitivity of the *rnpA49* mutation (32). As shown in Figure 3.1, the growth profile of the *rnpA49 ΔpcnB rph-1* mutant was similar to the *rnpA49 rph-1 rnpB*$^{+}$ strain, in which the M1 RNA was ectopically expressed off a plasmid. Interestingly, while the

suppression of temperature sensitivity of *rnpA49* mutant by M1 RNA does not work at 44 °C (32), the *rnpA49 ΔpcnB rph-1* mutant kept growing even at 44 °C (data not shown).

Level of short poly(A) tails increases dramatically in an RNase P mutant

Since PAP I is responsible for the majority of the polyadenylation in *E. coli*, we performed a poly(A) sizing assay to compare the distribution of poly(A) tails in various genetic backgrounds. Surprisingly, in the *rnpA49 rph-1* mutant (Figure 3.2, lane 3), there was a significant increase in the amount of short poly(A) tails (≤ 10 nt) compared to the *rph-1* strain (Figure 3.2, lane 2). As expected, majority of the poly(A) tails disappeared upon deletion of *pcnB* (Figure 2, lane 4), indicating that PAP I was the enzyme primarily responsible for addition of those poly(A) tails. Similar accumulation of the short poly(A) tails has been previously observed in the absence of RNase T and RNase PH, the primary 3′ – 5′ exoribonucleases involved in tRNA processing (Figure 3.2, lane 5; (21)). In the *rnpA49 Δrnt rph-1* triple mutant, the amount of short poly(A) tails increased even further (Figure 3.2, lane 6), suggesting that the *rnpA49* and *Δrnt* mutations contributed to the addition of poly(A) tails independently. Similar trends were observed for wild-type, *rnpA49* and *rnpA49 ΔpcnB* mutants (Figure 3.2, lanes 7-9).

Absence of PAP I affects the 3′ processing of certain tRNAs

Next we wanted to determine the exact location of the poly(A) tails added to the tRNA precursors. Therefore we analyzed the 5′ and 3′ sequences of the pre-tRNAs using cloning of 5′-3′ self-ligated transcripts. For *leuX* tRNA, all the clones (25/25) had extra

sequences at the 5′ end (Figure 3.3). It has been previously shown that RNase E cleaves non-specifically in the 5′ leader region of *leuX* precursor in the absence of RNase P, resulting in multiple 5′ ends (6). At the 3′ end, majority (22/25) of the clones had 1-4 untemplated A residues added post-transcriptionally. It must be noted that since the trailer sequence of *leuX* tRNA contains three A residues encoded chromosomally, it is not possible to determine the origin of the clone containing 1 A residue after the CCA. Only 2/25 clones were processed completely to the mature 3′ terminus of the *leuX* tRNA. In contrast, in the *rnpA49 ΔpcnB rph-1* mutant, while all the clones (23/23) were still deficient at the 5′ end, the 3′ ends of ~50% (12/23) clones were terminated at the CCA terminus. A similar effect was not observed for the *hisR* transcript. The 5′ cleavage sites were in agreement with previous data (1). In the *rnpA49 rph-1* strain, majority of the clones (26/28) had at least 2 extra nucleotides after the CCA determinant with 15/28 clones containing poly(A) tails of 1-3 nt in length. Unlike *leuX* tRNA, the 3′ end processing did not improve in the *rnpA49 ΔpcnB rph-1* mutant. Almost all the clones (24/26) still contained 2 extra residues.

Since both *leuX* and *hisR* transcripts contain long 5′ leaders in the absence of RNase P, we wanted to test if the length of the extra sequences would affect the processing of a tRNA primary transcript. Therefore we also tested *pheU/V* and *trpT* tRNAs. While the *pheU/V* tRNAs have been shown to be transcribed with a leader region of only 3-4 nucleotides (Bowden and Kushner, unpublished data), the monocistronic *trpT* tRNA is processed from the *rrnC* ribosomal RNA operons with a leader of ~4 nt. As

shown in Figure 3.4, the 3′ end processing of *pheU/V* tRNAs was significantly better even in the *rnpA49 rph-1* mutant. Over 50% (11/20) clones were processed to the mature 3′ termini even though almost all the clones (19/20) contained 1-4 extra nucleotides at the 5′ end. This suggests that the pre-tRNAs with short 5′ leaders are efficiently processed at the 3′ end.

Absence of PAP I leads to higher levels of aminoacylated tRNAs (In progress)

As shown in Figure 3.3, the processing of *leuX* transcript 3′ end improved significantly in the *rnpA49 ΔpcnB rph-1* mutant compared to the *rnpA49 rph-1* mutant. The improved processing should result in an increase in the pool of mature tRNA available for aminoacylation by the cognate aaRS enzymes. Correspondingly, the aminoacylation level for *leuX* tRNA should increase in the *rnpA49 ΔpcnB rph-1* mutant compared to the *rnpA49 rph-1* strain. Similarly, since majority of the *pheU/V* tRNAs were processed to the mature CCA terminus even in the *rnpA49 rph-1* mutant (Figure 3.4), the level of aminoacylated *phe* tRNAs should not reduce significantly compared to the *rph-1* strain. Therefore, we measured the *in vivo* level of aminoacylation of several tRNAs (Table 2).

Absence of Poly(A) polymerase I leads to increase in RNase T levels

It has been previously shown that over-expression of RNase T or RNase PH suppresses the toxicity associated with increased levels of PAP I. Higher cellular levels of the exonucleases restore growth rates and aminoacylation of tRNAs to wild-type levels. We hypothesized that the improved 3′ end processing of the pre-tRNAs in the absence of

PAP I could be due to an increase in the cellular levels of RNase T and/or RNase PH. We performed western analysis to determine the *in vivo* levels of RNase T in *rph-1*, *rnpA49 rph-1*, *ΔpcnB rph-1* and *rnpA49 ΔpcnB rph-1* genetic backgrounds, and RNase PH in *wild-type, rnpA49, ΔpcnB* and *rnpA49 ΔpcnB* mutants. As shown in Table 3.3, there was ~1.6- and 1.8-fold more RNase T in the *ΔpcnB rph-1* and *rnpA49 ΔpcnB rph-1* strains compared to the *rph-1* strain, respectively. This suggests that PAP I-mediated decay is involved in regulation of the *rnt* message. The levels of RNase PH remained unchanged (data not shown).

DISCUSSION

It has been historically presumed that tRNA precursor processing must be perfect at both the termini to allow aminoacylation. Here we show for the first time that the presence of few extra nucleotides at the 5′ end of tRNAs, which are processed to the mature CCA determinant at the 3′ termini, does not inhibit aminoacylation. Further, the lethality caused by an RNase P temperature sensitive allele is suppressed by the inactivation of poly(A) polymerase I. This is the first such evidence of extragenic suppression of the temperature sensitivity of an RNase P mutant allele.

It was previously shown that the absence of PAP I results in improved processing of tRNA precursors and faster growth rates in strains lacking RNase T, the primary 3′ – 5′ exoribonuclease. We wanted to test if inactivation of PAP I would result in a similar

improvement in the growth of an RNase P mutant carrying the *rnpA49* temperature sensitive allele. From the growth curve analysis (Figure 3.1), it is clear that the *rnpA49 ΔpcnB rph-1* strain continues growing at the otherwise non-permissive temperature of 42 °C even though the growth of *rnpA49 rph-1* mutant starts shutting down soon after the shift to the non-permissive temperature. A surprising observation was that this suppression of lethality of the temperature sensitive *rnpA49* allele worked even at 44 °C, where as the complementation of *rnpA49* by over-expression of M1 RNA has not been observed at that temperature (32). This suggests that the suppression due to inactivation of PAP I and over-expression of M1 RNA do not work through the same mechanisms. To understand the role of PAP I, we performed poly(A) sizing analysis to compare the total poly(A) levels in the *rnpA49 ΔpcnB rph-1* and *rnpA49 rph*-1mutants. As shown in Figure 3.2, there is significant accumulation of short poly(A) tails (≤ 10 nt) in the *rnpA49 rph-1* strain. Short poly(A) tails are typically associated with tRNAs and other stable RNAs such as 5S rRNA. The tails were absent in the *rnpA49 ΔpcnB rph-1* strain, confirming that PAP I was responsible for the addition of those short tails. We compared the increase in the short poly(A) tails in the *rnpA49 rph-1* strain to the previously observed accumulation in the absence of RNases T and PH. Since poly(A) sizing only gives a global profile, we decided to look at the polyadenylation profile of individual tRNA transcripts to determine the exact location and length of tails associated with specific tRNAs. For *leuX* tRNA, in the *rnpA49 rph-1* strain, majority of the clones were immature at both the termini, with 22/25 clones containing short (1-4 nt) tails at the 3′ end. However, removal of PAP I led to significantly improved processing at the 3′ end

and 12/23 clones were processed to the mature CCA determinant. These data suggest that in the absence of PAP I-mediated polyadenylation, the 3′ – 5′ exonucleases, primarily RNase T and RNase PH, are able to process the tRNA precursors to the mature 3′ end. In contrast, for *hisR* tRNA, absence of PAP I had no effect on the 3′ end processing. Majority of the clones (26/28 and 24/26) contained extra sequences after the CCA determinant in both the *rnpA49 rph-1* and *rnpA49 ΔpcnB rph-1* strains. One point to be considered about both the *leuX* and *hisR* tRNAs is the length of the extra sequences at the 5′ region. For both the tRNAs, inefficient RNase E cleavages generate pre-tRNAs with long 5′ leaders. To compare if the length of the 5′ leader has any effect on the processing of the 3′ end, we sequenced *phe* tRNAs. *pheU* and *pheV* primary transcripts have been shown to contain ~4 nt at the 5′ termini (Bowden and Kushner, unpublished data). Surprisingly, the 3′ end processing of *pheU*/*V* tRNAs was much more efficient, with ~50% (11/19, Figure 3.4) clones getting processed to the mature 3′ termini. Taken together, the sequencing data for *leuX*, *hisR* and *pheU*/V tRNAs suggests that the presence of short 5′ leaders does not interfere with 3′ processing and those pre-tRNAs are available for aminoacylation even if the 5′ ends aren't completely processed. For certain tRNAs with longer 5′ leaders, PAP I mediated-polyadenylation prevents RNase T and RNase PH from processing the 3′ ends. Inactivation of PAP I allows the exonucleases additional time. Additionally, in the absence of PAP I mediated regulation, the level of RNase T increased to ~1.8-fold in the *rnpA49 ΔpcnB rph-1* mutant, compared to *rph-1* strain. It has been previously shown that increased levels of RNase T can prevent the polyadenylation of tRNA precursors by rapidly processing the 3′ termini to

the mature CCA, which gets aminoacylated and is therefore no longer a substrate for PAP I. This explains the observation that 12/22 leuX clones were processed to the mature 3′ termini in the *rnpA49 ΔpcnB rph-1* strain compared to only 2/25 in the *rnpA49 rph-1* strain.

The data presented here raises the question about why RNase P is an essential enzyme. In a previous report, we examined whether ectopic expression of mature valine tRNAs could suppress the *rnpA49* temperature sensitivity. Although our hypothesis was proved incorrect, the fact that the aminoacylation levels for most tRNAs do not reduce significantly in the absence of RNase P (Table 3.2), suggests that the tRNA aminoacylation machinery has considerable tolerance for the presence of extra sequences at the 5′ ends of tRNA substrates. In *Nanoarchaeum equitans*, the only known organism lacking RNase P like activity, all the tRNAs are transcribed at their mature 5′ termini. This unique arrangement of promoter regions ensures that no 5′ end processing is required for tRNA maturation. This observation suggests that the essential function of RNase P is indeed its role in tRNA 5′ end processing. By Occam's razor, the likeliest explanation would be a global requirement of tRNA processing. Even though the aminoacylation level for any single tRNA does not reduce significantly in the absence of RNase P, the cumulative defect in tRNA processing is lethal to the cell.

TABLES AND FIGURES

Table 3.1: List of bacterial strains and plasmids used in this study

Strains	Genotype	Reference/ source
MG1693	*rph-1 thyA715*	*E. coli* Genetic Stock Center
SK2525	*rnpA49 thyA715 rph-1 rbsD296::*Tn*10* Tc^R	(2)
SK7988	*ΔpcnB thyA715 rph-1* Km^R	(33)
SK10148	*Δrnt thyA715 rph-1* Km^R	(2)
SK10153	*thyA715*	(21)
SK10297	*rnpA49 ΔpcnB thyA715 rph-1 rbsD296::*Tn*10* Tc^R Km^R	Mohanty & Kushner (unpublished data)
SK10521	*rnpA49 thyA715 rbsD296::*Tn*10* Tc^R	This study
SK10522	*rnpA49 ΔpcnB thyA715 rbsD296::*Tn*10* Tc^R Km^R	This study
SK10539	*rnpA49 thyA715 rph-1 rbsD296::*Tn*10* Tc^R / pAAK17	This study
SK10591	*ΔpcnB thyA715* Apr^R	Mohanty & Kushner (unpublished data)
SK10700	*rnpA49 Δrnt thyA715 rph-1 rbsD296::*Tn*10* Tc^R Km^R	Mohanty & Kushner (unpublished data)

Plasmids		
pBMK11	*pcnB*$^{+}$, Cm^{R}	(29)
pAAK17	pBMK11, *rnpB*$^{+}$,Cm^{R}	This study

Table 3.2: Aminoacylation level of tRNAs

tRNA isotype/genes	Percentage of aminoacylated tRNA		Number of nucleotides before mature 5′ terminus
	rph-1	***rnpA49 rph-1***	
Val/*valU,X,Y,T,Z*	39 ± 2	17 ± 6	9, ?[a]
Val/*valV,W*	38 ± 4	20 ± 3	?
Met/*metZ,W,V,Y*	83 ± 5	45 ± 3	?
Asn/*asnT,U,V,W*	65	58	1 – 4
Phe/*pheU,V*	73	46	1 – 4
Ser/*serV*	68 ± 8	18 ± 0.4	51
His/*hisR*	57 ± 2	21 ± 0.6	11
Pro/*proK,L,M*	61 ± 2	62 ± 2	2 – 4
Lys/*lysQ,T,V,W,Y,Z*	83 ± 8	52 ± 4	?
Leu/*leuW*	77 ± 4	74 ± 4	~5

[a]The five valine tRNAs are identical in sequence, but only the 5′ leader sequence for *valU* is confirmed through circularization experiments.

Table 3.3: Level of RNase T in various strains. Western analysis was performed using anti-RNase T polyclonal antibody. RNase T level n *rph-1* was set to 1. Relative Quantities represent averages of three independent determinations.

	Genotype			
	rph-1	***rnpA49 rph-1***	***ΔpcnB rph-1***	***rnpA49 ΔpcnB rph-1***
Relative Quantity (RQ)	1	1 ± 0.2	1.6 ± 0.2	1.8 ± 0.3

Figure 3.1: Growth curve analysis of various strains

All strains were initially grown at 30 °C until 50 Klett units above background, after which the cultures were shifted to 42 °C, denoted by the red dotted line. IPTG (350 μM) was added 30 min before shift. The cultures were diluted with pre-warmed LB/thymine or LB/thymine/IPTG to maintain them in exponential growth. The optical densities (OD) were measured with a Klett-Summerson Colorimeter (No. 42 Green filter).

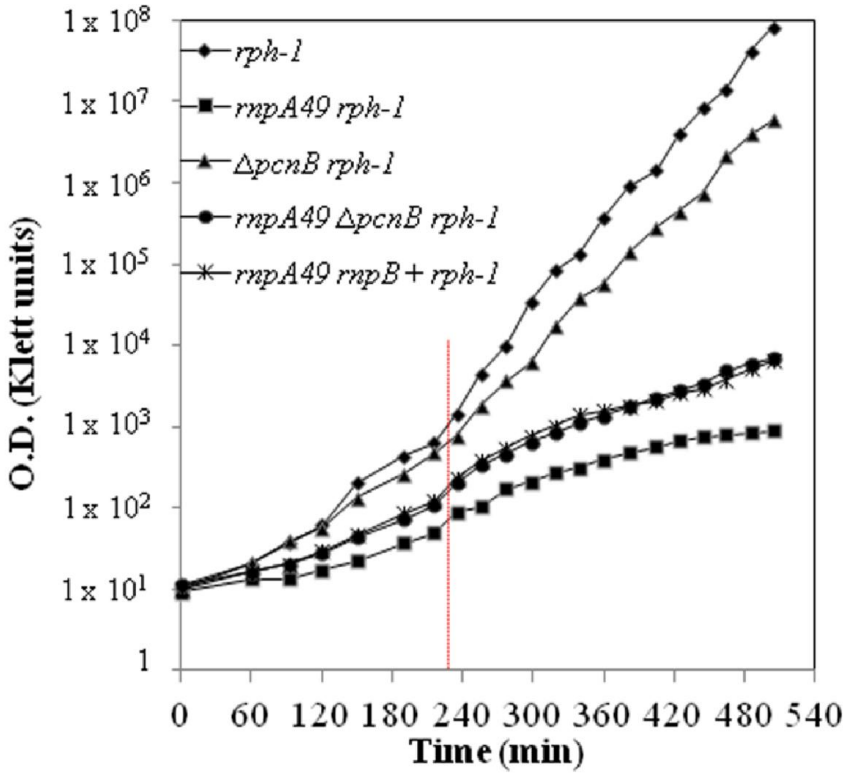


Figure 3.2 Poly(A) profile of RNase P and PAP I mutants

Distribution of poly(A) tails in various strains. The genotypes of the strains used are indicated on the top of the image. Total RNA from exponentially growing cultures was 3'-end-labeled with [32P]-pCp using T4 RNA ligase, followed by digestion with RNase A and RNase T1. Therefore the poly(A) tails are increased in length by 1 nt. 5'-end-labeled d(A) size standards are indicated to the left. The lane numbers are indicated at the bottom of the image.

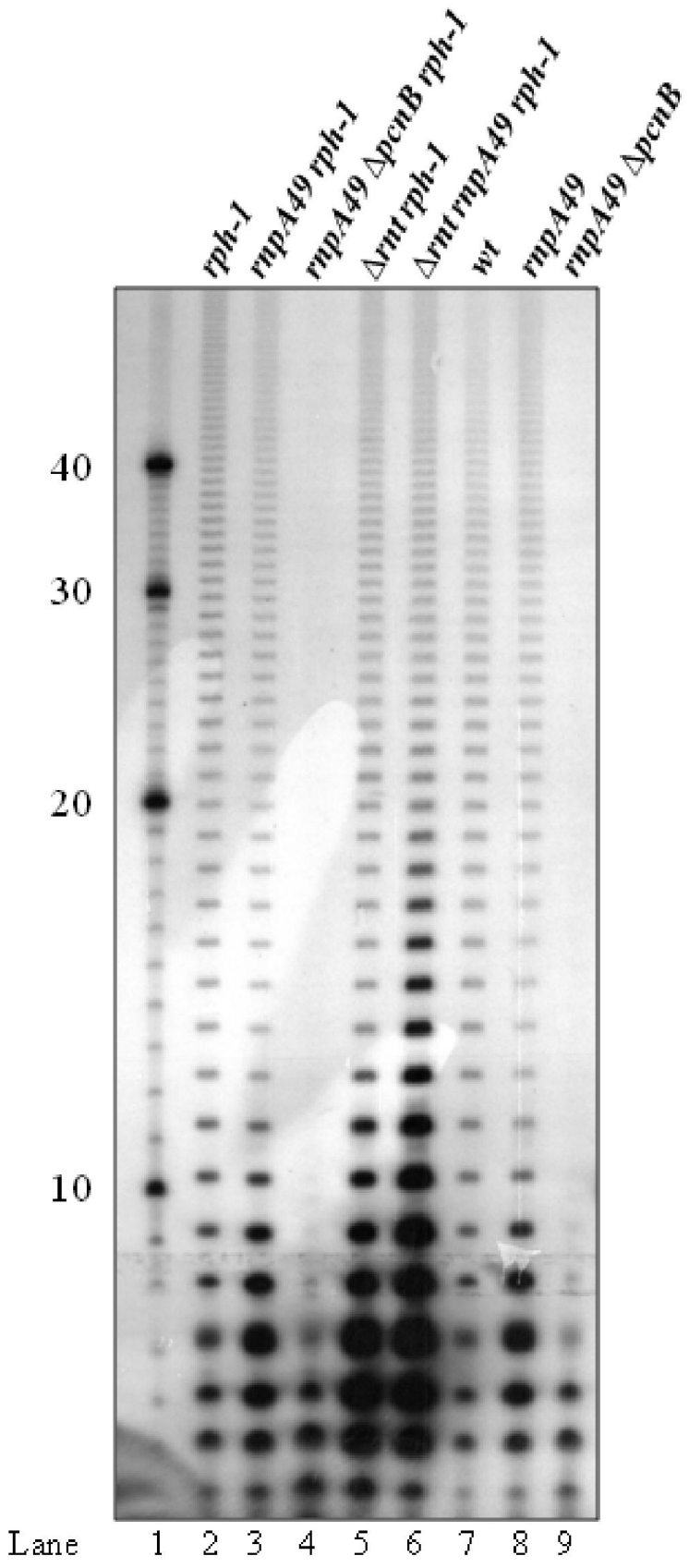

rph-1
rnpA49 rph-1
rnpA49 ΔpcnB rph-1
Δrnt rph-1
Δrnt rnpA49 rph-1
wt
rnpA49
rnpA49 ΔpcnB
40
30
20
10
Lane
1 2 3 4 5 6 7 8 9

Figure 3.3: Identification of 5′- and 3′-ends of *leuX* and *hisR* transcripts in various strains. The 5′ leader and 3′ trailer sequences of the respective tRNAs are shown next to schematic representations of *leuX* and *hisR* tRNAs (rectangles). The genotypes of the strains used for generating cDNA clones are indicated. The black downward arrows represent the 5′ and 3′ ends of sequences. The red upward arrows represent sites of untemplated poly(A) tails. The numbers directly above or below the arrows represent the number of independent clones identified with that specific end. The numbers in parenthesis represent the range of length of poly(A) tails at the 3′ sites.

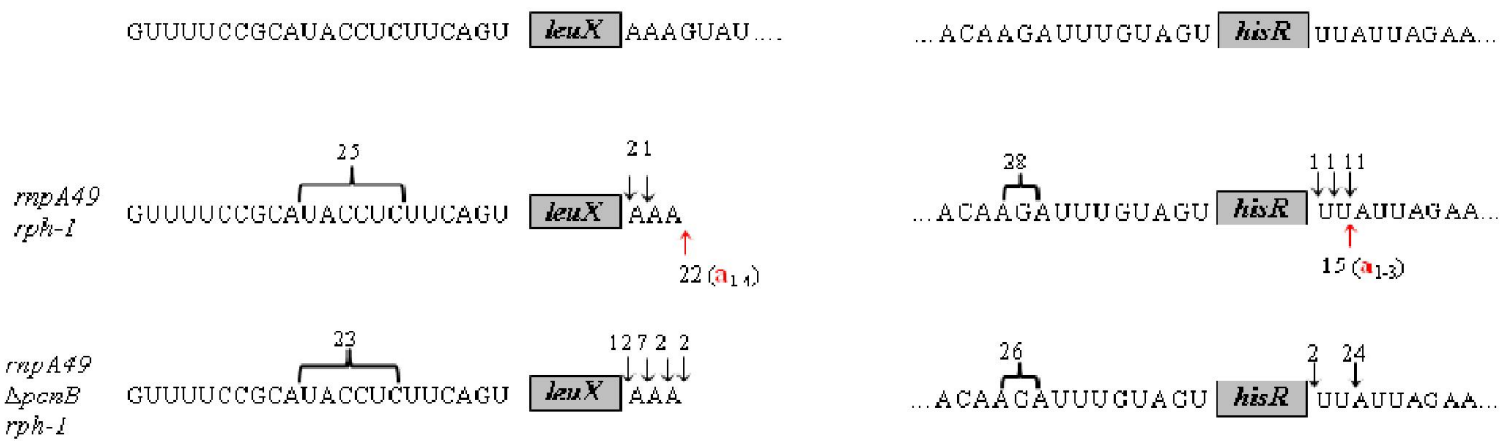
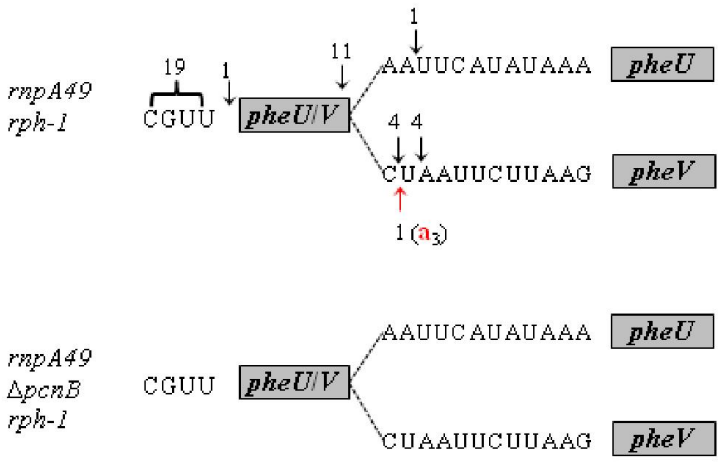

GUUUUCCGCAUACCUCUUCAGU leuX AAAGUAU....
...ACAAGAUUUGUAGU hisR UUAUUAGAA...
rnpA49
rph-1
25
21
GUUUUCCGCAUACCUCUUCAGU leuX AAA
22 (a1-4)
28
1 1 11
...ACAAGAUUUGUAGU hisR UUAUUAGAA...
15 (a1-3)
rnpA49
ΔpcnB
rph-1
23
12 7 2 2
GUUUUCCGCAUACCUCUUCAGU leuX AAA
26
2 24
...ACAACAUUUGUACU hisR UUAUUACAA...

Figure 3.4: Identification of 5′- and 3′-ends of *pheU/V* transcripts in various strains. The 5′ leader and 3′ trailer sequences are shown next to schematic representation of *pheU/V*. *pheU* and *pheV* 5′ leader region and mature sequences are identical. The 3′ end sequences are indicated separately. The genotypes of the strains used for generating cDNA clones are indicated. The black downward arrows represent the 5′ and 3′ ends of sequences. The red upward arrows represent sites of untemplated poly(A) tails. The numbers directly above or below the arrows represent the number of independent clones identified with that specific end. The numbers in parenthesis represent the range of length of poly(A) tails at the 3′ sites.



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CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Post-transcriptional processing of various RNA species is an integral means of maintaining and regulating cellular activities. All classes of RNA molecules undergo some extent of post-transcriptional processing during their life-time in the cell. tRNAs must undergo maturation to become functional molecules that can participate in their role in protein synthesis.

In Chapter 2, the processing of the valine tRNAs present in two polycistronic transcripts, *valU valX valY lysV* and *lysT valT lysW valZ lysY lysZ lysQ* was examined to better understand the role of RNase P in the cell. Our work demonstrated that RNase P was able to adeptly process all the individual tRNAs present in the two operons, including removing the Rho-independent transcription terminators. Surprisingly, the processing proceeds in a general 3′ – 5′ direction with the Rho-independent terminator being removed first. Subsequently, starting at the distal end, one tRNA at a time is cleaved at the mature 5′ terminus. The data also demonstrates that RNase P can remove Rho- independent terminators very efficiently

An interesting observation was that despite the presence of long intergenic regions, none of the other endoribonucleases in *E. coli* including RNase E played any

significant role in the processing of the two operons. Even though RNase E was able to inefficiently remove the Rho-independent terminator after the *valU* operon and cleave within the large intergenic spacers for the *lysT* operon, these cleavages were only observed in the absence of RNase P. This suggests that RNase E merely plays a backup role in the processing of these large polycistronic operons.

Surprisingly, valine tRNAs were preferentially processed by RNase PH at their 3′ end. Typically, for most tRNAs, RNase T plays a more prominent role in the 3′ end processing (1). We have now seen a similar effect with *leuW* tRNA (Agrawal and Kushner, unpublished data). However, the improved processing did not lead to any increase in the aminoacylation levels of valine tRNA. Also, our data shows that the *rnpA49* temperature sensitive mutant allele of the C5 protein that has been most commonly used to study the biological role of RNase P is considerably defective even at the permissive temperature (2).

The aminoacylation data presented in Chapter 2 shows that the level of aminoacylated valine tRNAs was reduced by only ~2 fold in the absence of RNase P. This suggests that the presence of a few extra nucleotides at the 5′ termini of tRNAs might not be completely inhibitory to their aminoacylation, as has been previously suggested.

In Chapter 3, we presented our work studying the interaction between RNase P and poly(A) polymerase I. We demonstrated that inactivation of PAP I results in suppression of the lethality associated with the RNase P temperature sensitive mutants.

Removal of PAP I has been previously shown to improve the tRNA processing and growth rate of strains lacking RNase T and RNase PH (3). However, the improvement in growth of the *rnpA49 ΔpcnB rph-1* mutant at the non-permissive temperatures was still quite surprising. While RNase T/ RNase PH and PAP I both compete for the 3′ ends of tRNA substrates, RNase P is primarily involved in the 5′ end maturation, and shouldn't be really affected by the 3′ activities.

Our data shows that there is significant accumulation of short poly(A) tails (~2-10 nt), typically associated with tRNAs, in the absence of RNase P. These tails are primarily present on pre-tRNAs with long 5′ leaders in the absence of RNase P. tRNAs which have short leaders either due to processing by other endonucleases or because of a proximal transcription initiation site, the 3′ processing isn't affected significantly. Removal of PAP I allows the 3′ exonucleases additional opportunity to process the pre-tRNAs to their mature CCA termini. Also, PAP I is involved in the regulation of the *rnt* message, and in the absence of PAP I there is a ~1.8 fold increase in the RNase T levels. Increased cellular levels of RNase T have been shown to negate the effects of polyadenylation of tRNA precursors (4). Improved processing of the 3′ ends of these tRNAs would result in a greater pool of mature tRNAs being available for aminoacylation, which would lead to an improvement in protein synthesis, and that in turn would increase the growth rate.

Several interesting questions arise based on the work done in Chapters 2 and 3. Why does RNase P process the *valU* and *lysT* operons in a general 3′ – 5′ direction? How does RNase P remove Rho-independent transcription terminators? How does the RNase

P holoenzyme bind to these large polycistronic transcripts? Why isn't RNase E more significantly involved in the processing of the *valU* and *lysT* operons? Why is the aminoacylation level of valine tRNAs low even is wild-type *E. coli*? What dictates the tRNA substrate selection for RNase T and RNase PH and why are the valine tRNAs preferentially processed by RNase PH? If the activities of RNase T and RNase PH are not completely substitutable, why do several wild-type *E. coli* strains lack a functional RNase PH enzyme? Is there a specific length of 5′ leaders that interferes with the 3′ end processing enzymes as well as the aminoacyl tRNA synthetases? How do aminoacylated tRNAs containing 5′ leaders fit into the ribosome during protein synthesis? How does the competition between 3′ end processing exonucleases and polyadenylation activities work in other prokaryotes which lack a canonical poly(A) polymerase? Does tRNA processing efficiency contribute to the growth rate of other prokaryotes?

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