Trends in Genetics

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REVIEWS

Review

New Insights into the Relationship between tRNA Processing and Polyadenylation in *Escherichia coli*

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Recent studies suggest that poly(A) polymerase I (PAP I)-mediated polyadenylation in *Escherichia coli* is highly prevalent among mRNAs as well as tRNA precursors. Primary tRNA transcripts are initially processed endonucleolytically to generate pre-tRNA species, which undergo 5′-end maturation by the ribozyme RNase P. Subsequently, a group of 3′ → 5′ exonucleases mature the 3′ ends of the majority of tRNAs with few exceptions. PAP I competes with the 3′ → 5′ exonucleases for pre-tRNA substrates adding short poly(A) tails, which not only modulate the stability of the pre-tRNAs, but also regulate the availability of functional tRNAs. In this review, we discuss the recent discoveries of new tRNA processing pathways and the implications of polyadenylation in tRNA metabolism in *E. coli*.

Highlights

The majority of transcripts in *E. coli* undergo post-transcriptional addition of poly(A) tails by PAP I. Recent studies suggest that *in vivo* 79/86 pre-tRNA species undergo PAP I-dependent polyadenylation to some extent prior to final 3′ end maturation.

In wild-type *E. coli* mature tRNAs are generally not polyadenylated due to higher levels of 3′ → 5′ tRNA-processing exonucleases compared with PAP I, which favors aminoacylation by aminoacyl-tRNA synthetases.

Unregulated PAP I expression results in the polyadenylation of mature tRNAs, hindering aminoacylation by aminoacyl-tRNA synthetases, leading to inhibition of protein synthesis and cell death.

Structure and Organization of tRNA Genes

tRNAs are essential in all living organisms, based on their role as adapter molecules that present the correct amino acids to the translation machinery so that the information contained in

mRNAs can be accurately decoded. To date, over 6000 bacterial genomes have been sequenced and more than 363 000 tRNA genes have been annotated [1]. However, our understanding of tRNA processing is most advanced in the model organism *E. coli.* It contains 86 tRNA genes that encode 40 mature tRNA isotypes associated with the standard 20 amino acids. The large majority of the tRNA genes ($n = 64$) are expressed from **polycistronic operons** (see Glossary) containing identical or unrelated tRNAs, rRNA, or protein encoding genes (Table 1). The remainder of the tRNAs are expressed from **monocistronic genes**. While four tRNAs ($tRNA^{Cys}$, $tRNA^{His}$, $tRNA^{Sel}$, and $tRNA^{Trp}$) are represented by only one gene each, the rest of the tRNAs are present in more than one copy, either in the same or different operons. There is a strong correlation between tRNA abundance and codon usage at a number of different growth rates [2,3]. Unlike eukaryotes, all the tRNA genes in *E. coli* lack introns. However, all primary tRNA transcripts, irrespective of the organism, require extensive post-transcriptional processing (Box 1).

tRNA Processing Pathways

For many years, it was thought that all *E. coli* tRNAs were matured via a common processing pathway following a 'one shoe fits all' model [4]. The observation of reduced RNase P activity on pre-tRNAs with long precursor sequences [5] and in the absence of RNase E led to the suggestion that an initial RNase E cleavage is required for RNase P to generate the mature 5′ terminus [6–8]. Moreover, the involvement of RNase E in the initiation of tRNA processing has been proposed to be the basis of its essential function [6,8,9]. Thus, the consensus pathway that evolved over time was that the initial processing of all primary tRNA transcripts, both mono- and polycistronic, involved RNase E to generate pre-tRNAs containing a few extra nucle-

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Table 1. Organization of tRNA Genes in the MG1655 *E. coli* Genome[a]

Monocistronic tRNA genes
argU, argW, aspV, asnV, asnU, asnT, glyU, ileX, ileY, leuX, metY, pheU, pheV, proK, proL, selC, serU, serW, serX, serT, thrW
Polycistronic tRNA operons
alaW alaX, argX hisR leuT proM, glyV glyX glyY, glyW cysT leuZ, leuQ leuP leuV, lysT valT lysW valZ lysY lysZ lysQ, metT leuW glnU glnW metU glnV glnX, metZ metW metV, serV argV argY argZ argQ, valU valX valY lysV, valV valW
Polycistronic operons containing tRNA and mRNA genes
secG ***leuU***, ***thrU tyrU glyT thrT*** *tufB*, ***tyrT tyrV*** *rttR*
Polycistronic tRNA genes associated with rRNA operons
rrsA ***ileT alaT*** *rrlA rrfA*, *rrsB* ***gltT*** *rrlB rrfB*, *rrsC* ***gltU*** *rrlC rrfC* ***aspT trpT***, *rrsD* ***ileU alaU*** *rrlD rrfD* ***thrV*** *rrfF*, *rrsE* ***gltV*** *rrlE rrfE*, *rrsG* ***gltW*** *rrlG rrfG*, *rrsH* ***ileV alaV*** *rrlH rrfH* ***aspU***

[a]Bold letters: tRNA genes

otides at both the 5′- and 3′-ends (Figure 1A). Subsequently, the pre-tRNAs were matured at the 5′ end by RNase P and at the 3′ end by multiple 3′ → 5′ **exoribonucleases**, such as RNase T, RNase PH, RNase D, and RNase BN (Box 2). Notable tRNA operons processed by this pathway are *glyW cysT leuZ* and *argX hisR leuT proM* (Figure 1A) [6,8].

However, it was noticed [8] that there were significant differences in the cleavage specificity of RNase E for many of the primary tRNA transcripts. In addition, it was observed [10] that a number of tRNA operons lack predicted RNase E cleavage sites in the intergenic spacer regions, suggesting the involvement of ribonucleases other than RNase E in separation of these tRNAs. In fact, the

Box 1. Primary tRNA Transcripts Require Extensive Post-transcriptional Processing

There are ~375 000 functional tRNA molecules in an exponentially growing *E. coli* cell [59]. For a tRNA to be functional (i.e. to be able to be aminoacylated), it must have the nucleotides CCA at its immediate 3′ terminus [60]. In many organisms, including eukaryotes and numerous bacterial species, the CCA determinant is added post-transcriptionally by the CCA-adding enzyme tRNA nucleotidyltransferase. The exact role of tRNA nucleotidyltransferase in the Gram-negative bacterium *E. coli* is not fully understood, since the CCA determinant is encoded in the DNA for every tRNA. It has been argued that the enzyme is needed for tRNA 3′-end repair [61,62]. Surprisingly, the parasitic bacteria *Mycoplasma* and *Ureaplasma* (six genomes) also encodes the CCA triplet for all tRNA genes, but lack both tRNA nucleotidyltransferase and PAP [63].

Even though all *E. coli* tRNAs have encoded CCA determinants, their primary transcripts must undergo extensive post-transcriptional processing in order to be functional. In fact, the processing of primary tRNA transcripts to produce mature tRNAs involves a significant number of ribonucleases with distinct roles. In almost all cases, **endoribonucleases** (RNase E and/or RNase P) cleave the polycistonic tRNA transcripts to generate separate pre-tRNAs that contain extra nucleotides at both their 5′ and 3′ ends. Subsequently, additional enzymes (both endo- and exoribonucleases) are used to generate the mature 3′ and 5′ termini.

To date, RNase P is the only known enzyme that is responsible for generating the mature 5′ ends of all tRNAs in all kingdoms of life. The sole exception is an obligatory parasitic archaeon *Nanoarchaeum equitans* where the primary tRNA transcripts start at the nucleotide that is the mature 5′ terminus [64]. Inactivation of RNase P leads to accumulation of 5′-extended tRNAs and nonviability in *E. coli.* Considering that many tRNAs with 5′ extensions can still be aminoacylated [10,65–67], the exact reason for unviability in the absence of RNase P has yet to be determined.

In *E. coli*, the majority of tRNA 3′-end maturation involves one or more of the major 3′ ⊠ 5′ exonucleases (RNase T, RNase PH, RNase D, RNase BN, or RNase II) [4]. However, the 3′ ends of some tRNAs are matured by the endoribonuclease RNase E in a single step [19]. All these processing events are distinct from the many post-transcriptional modifications that generate the various altered nucleotides that are found in functional tRNAs.

Glossary

Aminoacylation: addition of an amino acid to the mature 3′ end of a tRNA.
Endoribonuclease: enzyme that cleaves RNA molecules at internal sites.
Exoribonuclease: enzyme that degrades RNA either from 3′or 5′ end one nucleotide at a time.
Homopolymeric poly(A) tail: poly (A) tail containing all A nucleotides.
Monocistronic gene: single transcriptional unit (gene).
Poly(A) polymerase I (PAP I): PAP of *E. coli* encoded by *pcnB* gene.
Polycistronic operon: group of genes that are transcribed as a single mRNA.
Polynucleotide tail: post-transcriptionally added tails at the 3′ end of a transcript containing all four nucleotides with mostly A nucleotides.
Rho-dependent transcription terminators: Rho factor is a prokaryotic protein that interacts with RNA polymerase to promote the termination of many prokaryotic transcripts.
Rho-independent transcription terminator: also known as intrinsic termination. Some prokaryotic transcripts do not require Rho factor for termination. Instead, these transcripts are terminated through a mechanism involving a terminal stem-loop structure followed by a U-rich sequences on the mRNA that interacts with RNA polymerase.

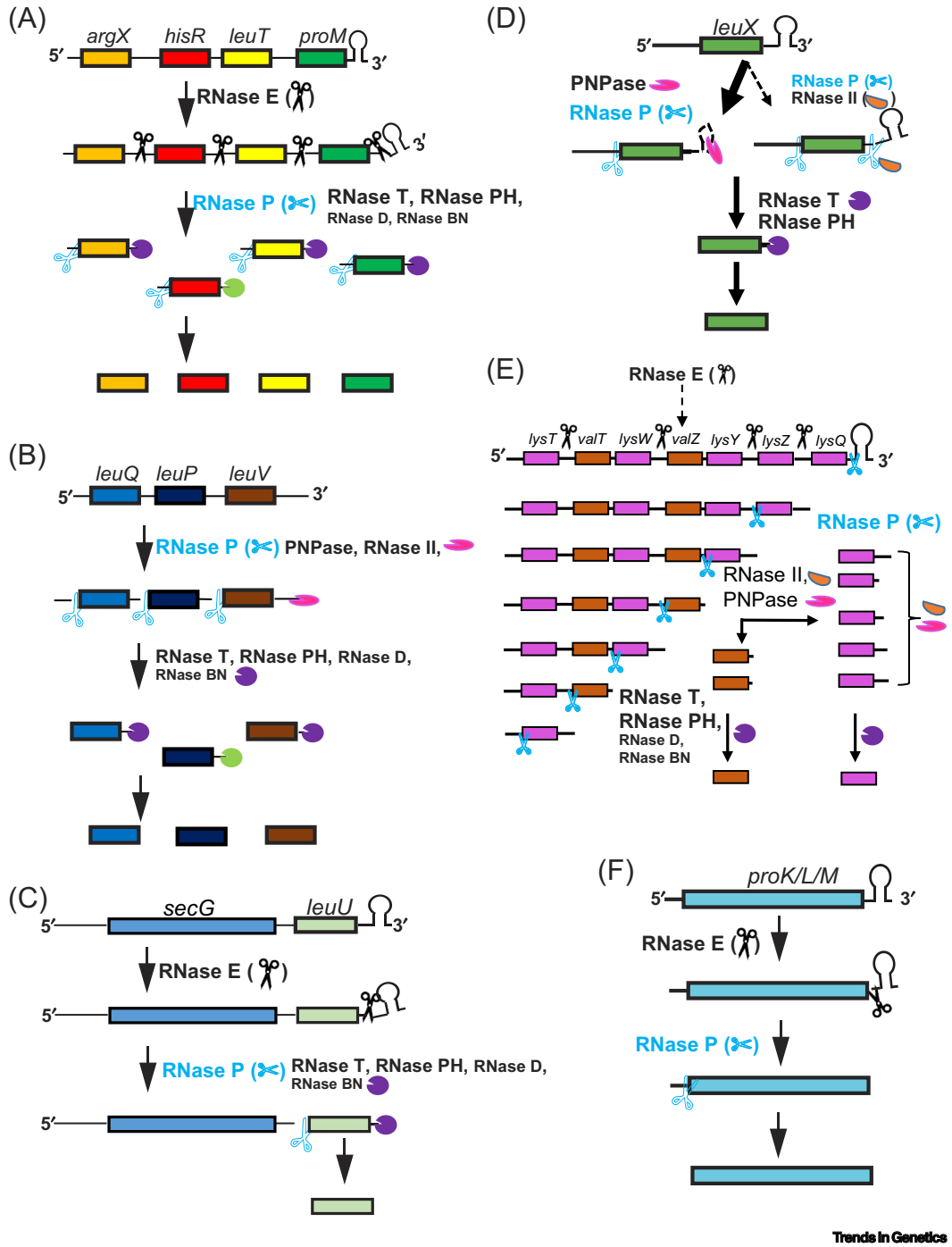

Figure 1. tRNA Processing Pathways in *Escherichia coli* (Not Drawn to Scale). (A). RNase E initiates the processing of the *argX hisR leuT proM* polycistronic operon by removing the Rho-independent transcription terminator and separating the pre-tRNAs [8]. Subsequently, RNase P cleaves at the 5′-termini and RNase T and/or RNase PH removes the 3′-extension to expose the CCA determinant. (B) RNase P initiates the processing of the *leuQ leuP leuV* polycistronic operon by separating pre-tRNAs with direct cleavages at the mature 5′ ends while the 3′ Rho-dependent terminator is shortened by a combination of PNPase and RNase II [10]. Finally, RNase T and/or RNase PH matures the 3′ ends. (C) In case of the *secG leuU* polycistronic transcript, once the Rho-independent transcription terminator is

(Figure legend continued at the bottom of the next page.)

Box 2. Processing the 3′ Termini of Pre-tRNAs

Endonucleolytically generated pre-tRNAs very often contain nucleotides upstream of mature 5′ end and beyond CCA trinucleotide at the 3′ end. The removal of the extra nucleotides from the 3′ termini of most pre-tRNAs involves a hierarchy of at least five 3′ → 5′ exonucleases (RNase T, RNase PH, RNase D, RNase BN, and RNase II) [55]. RNase T and RNase PH are considered to be the two primary exoribonucleases, which carry out the majority of tRNA 3′-end processing. RNase T is the most effective in tRNA 3′-end processing, but is inhibited by the presence of C residues at its active site [62]. For those tRNAs that have a C residue immediately downstream of the CCA determinant, RNase PH is primarily responsible for the final processing of its 3′ terminus [11,62] (Table 2). Several additional tRNAs where CCA determinants are followed immediately by C residues (Table 3) are most likely also dependent on RNase PH and await experimental confirmation. If both RNase T and RNase PH are missing, cells grow more slowly but mature tRNAs are still generated through a combination of RNase D, RNase BN/Z, and RNase II [18]. The loss of RNase D and RNase BN/Z, in the presence of RNase T and RNase PH, has no effect on either growth or tRNA 3′-end processing, but the absence of all five exonucleases is a synthetic lethal [68].

Many of the tRNA 3′-end processing exonucleases act on multiple RNA substrates [69]. While RNase PH and RNase D participate only in tRNA 3′-end processing, RNase T participates in tRNA 3′-end processing, 23S, and 5S rRNA 3′-end maturation with equal preference [70,71]. RNase BN, initially identified as a 3′→ 5′ exonuclease involved in tRNA 3′-end maturation [72], has now been shown also to have endonuclease activity and is called RNase Z [73]. Unlike eukaryotic RNase Z, which cleaves tRNA precursors endonucleolytically at the 3′ end to generate substrates for tRNA nucleotidyltransferase to add the CCA determinant [74], *E. coli* RNase Z does not use pre-tRNA as a substrate [75]. Instead, RNase Z functions primarily in mRNA decay [76]. RNase II is primarily involved in mRNA decay [77,78].

processing of the transcripts of the *valV valW* and *leuQ leuP leuV* operons was shown to be initiated exclusively by RNase P [10] (Figure 1B). It should be noted that these two operons are terminated by **Rho-dependent transcription terminators** unlike the *glyW* and *argX* operons, which are terminated by **Rho-independent transcription terminators** where the processing is initiated by RNase E. Subsequently, RNase P also was shown to separate all the pre-tRNAs for some transcripts terminated with Rho-independent transcription terminators, such as the *secG leuU* and *valU valX valY lysV* operons, but only after the removal of the transcription terminator by RNase E (Figure 1C) [11,12].

However, not all Rho-independent transcription terminators are removed by RNase E. In the case of the *leuX* monocistronic transcript, polynucleotide phosphorylase (PNPase), a 3′ → 5′ exonuclease, is primarily responsible for the removal of the terminator from ~90% of the transcripts, while RNase P removes the terminator from rest of the transcripts (Figure 1D) [13]. Another type of processing has been described for the *lysT valT lysW valZ lysY lysZ lysQ* operon, which is also terminated with a Rho-independent transcription terminator [11]. In this case, RNase P processes the operon in the 3′ → 5′ direction starting with the removal of the Rho-independent transcription terminator (Figure 1E) [11]. In the absence of RNase P, RNase E can cleave in the long intergenic regions (Figure 1E) [11].

removed primarily by RNase E, RNase P cleaves at the *leuU* mature 5′ end and RNase T and/or RNase PH matures the 3′ end [12]. (D) Processing of the monocistronic *leuX* transcript [13] involves exonucleolytic removal of the Rho-independent transcription terminator by PNPase for ~90% of the transcripts (thick downward arrow). The Rho-independent transcription terminators in ~10% of the transcripts are removed endonucleolytically by RNase P followed by shortening of the 3′ end by RNase II (thin dashed downward arrow). Both pre-tRNAs are processed further by RNase P at the mature 5′ terminus and by RNase T and/or RNase PH at the 3′ terminus. (E) The processing of the *lysT* operon [11] is initiated by RNase P by removing the Rho-independent transcription terminator. Subsequently, RNase P cleaves at the mature 5′ end of each of the upstream tRNAs sequentially from the 3′ end of the operon releasing pre-tRNAs with immature 3′ ends ranging from 2 to 150 nucleotides. The longer 3′ extensions are shortened by a combination of PNPase and RNase II before RNase T and/or RNase PH mature the 3′ ends. RNase D and/or RNase BN (in small letters) also participate in the 3′-end maturation, albeit slowly, in all the above processing pathways only in the absence of the major exonucleases RNase T and RNase PH. In the absence of RNase P, RNase E can cleave at the indicated sites separating the pre-tRNAs (dashed downward arrow). (F) Processing of the *proK*, *proL*, and *proM* transcripts [19] involves RNase E removing the Rho-independent transcription terminator in one step to generate the mature 3′ terminus without the need of any of the 3′ → 5′ exonucleases. RNase P cleaves at the mature 5′ end. Abbreviations: PNPase, polynucleotide phosphorylase; RNase, ribonuclease.

The cleavage specificity of RNase E on tRNA substrates is still poorly understood. RNase E is believed to cleave at an AU-rich sequence element (AUE) [14–17]. In fact, bacteria containing RNase E have been shown to have conserved AUE elements downstream of each tRNA [7]. Thus, most of the tRNA processing pathways described above generate pre-tRNAs that require one or more of the 3′→ 5′ exoribonucleases for maturation of the 3′ ends, since RNase E endonucleolytic cleavages of primary tRNA transcripts normally occurs 1–3 nucleotides (nts) downstream of the CCA terminus at an AUE element [6,8].

In contrast, the *proK*, *proL*, *proM*, *metV, metW*, *metY*, and *metZ* tRNAs are matured exclusively by RNase E using a one-step cleavage that exposes the CCA terminus without utilizing any of the 3′ → 5′ exonucleases [18] (Figure 1F). This result was surprising, since RNase E removes the Rho-independent transcription terminators associated with *proK*, *pheU*, and *leuU* primary transcripts differently, even though all these transcripts have similar AUE elements. Furthermore, while the downstream sequences of *proL* and *leuX* are similar, RNase E cleaves immediately downstream of the CCA of *proL* but *leuX* is not a substrate [19]. Instead, PNPase removes the Rho-independent transcription terminator of *leuX* [13]. With the exception of *proK*, *proL*, *proM*, *metV, metW*, *metY*, and *metZ* tRNAs, all other tRNAs require 3′-end maturation by 3′→5′ exonucleases (Box 2).

Discovery of the Role of Polyadenylation in tRNA Processing

Polyadenylation in bacteria received very little or no attention for many years in spite of the fact that the first poly(A) polymerase (PAP) was discovered in *E. coli* in 1962 [20,21]. However, since the identification of *pcnB* as the structural gene for **PAP I** 30 years later [22], significant progress has been made in identifying polyadenylation targets as well as its role in bacteria [13]. Genome-wide analysis in *E. coli* using oligo(dT)-dependent cDNAs derived from oligo(dT)-affinity purified transcripts suggested that the transcripts derived from the majority of the open reading frames (ORFs) are polyadenylated to some extent under exponentially growing conditions [23]. However, a recent RNA sequencing (RNAseq) study using the 5′-tag rapid amplification of cDNA ends (tagRACE) approach has suggested that there are fewer polyadenylation targets in *E. coli* [24]. It should be noted that ligation-based techniques to determine the polyadenylation state of transcripts have been extremely inefficient in our hands.

Unlike in eukaryotes, only a limited fraction of bacterial mRNAs are polyadenylated at any given time. However, the actual extent of polyadenylation is known only for a few of the transcripts. These data suggest that the level of polyadenylation for any particular transcript is not dependent on either its size or abundance [25]. The level of polyadenylation can vary from as low as 0.4% to as high as 10%. Although these experiments have provided important insights into bacterial mRNA metabolism [25–27], its role in tRNA metabolism was still poorly understood.

The *E. coli* K12 strains MG1693 and W3110, which are widely used as the reference strains, are known to contain the *rph-1* allele, which has a single base pair deletion in the C-terminal region of the *rph* gene, resulting in a truncated RNase PH protein that has no 3′ → 5′ exonuclease activity [28,29]. The significance of the *rph-1* mutation was not realized until it was shown that many pre-tRNAs containing one or more C residues downstream of the CCA determinant were not substrates for RNase T, but were dependent on RNase PH for their final 3′-end processing (Table 2) [30]. Thus, the pre-tRNAs that are not fully processed in *rph-1* strains are prevented from being **aminoacylated** and become potential targets for polyadenylation. $tRNA^{Tyr}$ was one of the first tRNAs found to be polyadenylated in multiple exonuclease mutants [31]. Subsequently, using a temperature sensitive mutant of $tRNA^{Trp}$, it was proposed that defective tRNA precursors are polyadenylated and degraded as part of a general quality control pathway [32].

Table 2. tRNA 3′ Ends Processed by RNase PH

tRNAs	3′ nucleotides following the CCA determinant	Refs
cysT	CU	[18]
pheV	CU	[29]
valV	UC	[56]
valE, *valX*, *valY*, *valZ*	CU	[11,56]

Table 3. tRNA 3′ Ends Containing a C Nucleotide Immediately Downstream of CCA

tRNAs	3′ nucleotides following the CCA determinant
aspT, *valT*	CC
aspT, *aspU*, *glnT*, *glnU*, *glnV*, *glnW*, *serU*, *thrT*, *thrV*	CU
alaT, *alaU*, *alaV*, *glnW*, *thrU*, *tyrV*	UC
glnX	CA
leuT	CG
valU	AC

Table 4. Polyadenylated tRNAs in *E. coli*

tRNAs[a]	No. of tRNA genes	Genotypes	Refs
Arg2	4	*Δrnt rph-1*	[18]
Asn	4	*Δrnt rph-1*	[18]
Cys	1	Wild type, *rph-1*, *Δrnt rph-1*	[12,18]
Glu2	4	*Δrnt rph-1*	[18]
His	1	Wild type, *rph-1*, *Δrnt rph-1*	[12,18]
Leu1	4	*Δrnt rph-1*	[18]
Leu2	1	*rph-1*, *Δrnt rph-1*	[12,18]
Leu3	1	*Δrnt rph-1*	[18]
Leu4	1	*Δrnt rph-1*	[18]
Leu5	1	*rph-1*, *Δrnt rph-1*	[13,18]
Lys	6	*Δrnt rph-1*	[18]
Met m	2	*Δrnt rph-1*	[18]
Phe	2	*Δrnt rph-1*	[18,29]
Ser1	1	*Δrnt rph-1*	[18]
Ser3	1	*Δrnt rph-1*	[18]
Ser5	2	*Δrnt rph-1*	[18]
Tyr1	1	*Δrnt Δrnd Δrbn rph-1*	[31]
Tyr2	2	*Δrnt Δrnd Δrbn rph-1*	[31]
Val1	5	*Δrnt rph-1*	[18]
Val2A	1	*Δrnt rph-1*	[18]
Val2B	1	*Δrnt rph-1*	[18]

[a]Length of poly(A) tails associated with these tRNAs was in the range of 1–5 nt based on either northern analysis or sequencing of cDNAs.

The demonstration of polyadenylation of pre-tRNAs (Table 4) in both a wild-type and various exonuclease mutants [12,13,18,29,33] suggested that polyadenylation plays a more significant role in tRNA metabolism.

The prevalence of polyadenylation among specific tRNAs in *E. coli* at any given time is still unknown. However, based on cloning and sequencing data [18,25], 8–23% of *hisR*, *cyst*, and *leuX* pre-tRNAs were reported to have poly(A) tails in a wild-type strain. The percentage of poly (A) tails increased (20–44%) in the *rph-1* mutant, which was consistent with the role played by RNase PH as a 3′→5′ tRNA 3′-end processing exonuclease. About 44% of *pheU* and *pheV* pre-tRNAs were reported to contain poly(A) tails in the *rph-1* mutant where the *pheV* transcript is dependent on RNase PH for final 3′-end maturation [29]. In the absence of both RNase PH and RNase T, the percentage of transcripts with poly(A) tails increased to ~80% for *hisR* and ~53% for *cysT*. However, no poly(A) tails were reported at the 3′-mature termini of any of the above transcripts either in the *rph-1* mutant or the wild-type control. Interestingly, 8–13% of the *hisR* and *cysT* tRNAs contained poly(A) tails at the mature 3′ termini in the *rph-1 Δrnt* double mutant [18].

Overall, 79/86 pre-tRNAs are extensively polyadenylated in the absence of both RNase T and RNase PH [18], indicating that these ribonucleases are major impediments to tRNA polyadenylation. This observation provided a possible explanation for why deregulation of PAP I synthesis in *E. coli* leads to a significant increase in toxicity [34]. Specifically, there is an ongoing competition between

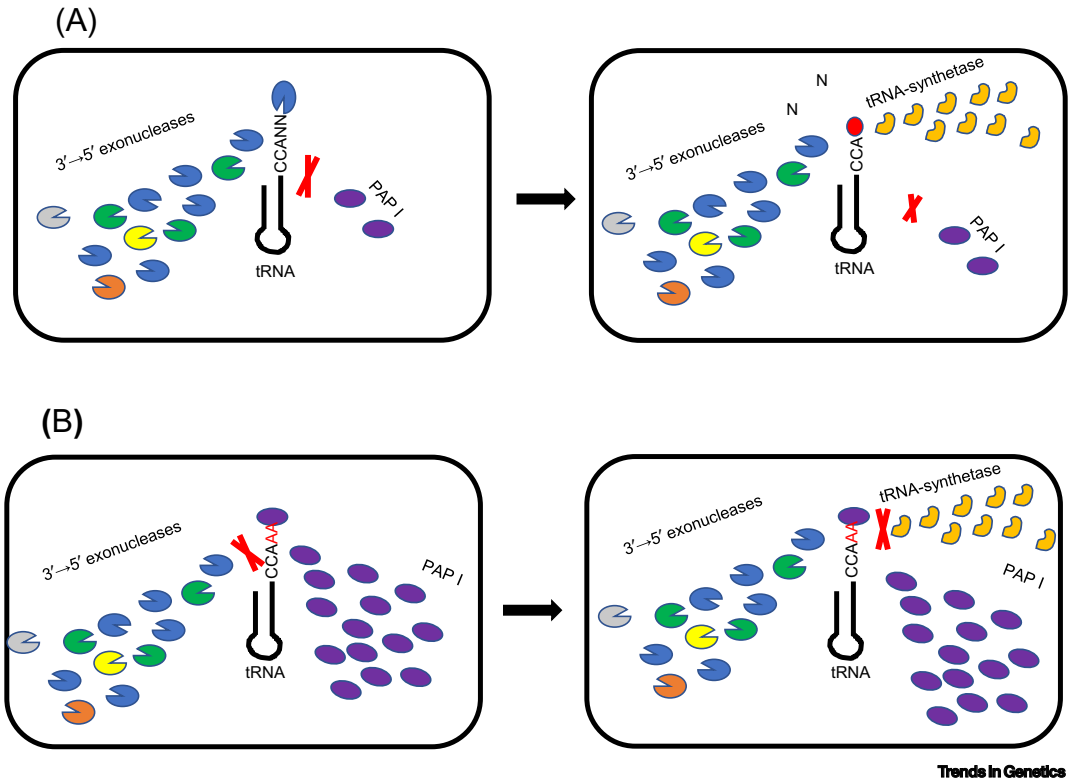

Figure 2. Graphical Presentation (Not Drawn to Scale) of Competition between tRNA 3′-End Processing 3′→5′ Exoribonucleases (RNase T, RNase PH, RNase D, RNase BN, and RNase II), PAP I and Aminoacyl tRNA-Synthetases for the Pre-tRNA/tRNA Substrates. (A) pre-tRNAs are matured at the 3′ ends at a faster rate by a higher level of exonucleases compared with PAP I. These tRNAs immediately become substrates for aminoacyl tRNA-synthetases, which add an amino acid (red circle) and are not available (red cross) for polyadenylation by PAP I. (B) Increased levels of PAP I outcompete the 3′ → 5′ exonucleases for the pre-tRNA/tRNA substrates and adds poly(A) tails making them unsuitable (red cross) for aminoacylation. Abbreviations: PAP I, poly(A) polymerase I.

the activities of tRNA 3′-end processing ribonucleases and polyadenylation by PAP I. Because of the significantly higher levels of 3′-end processing enzymes compared with PAP I in wild-type cells, the majority of tRNA 3′ ends are matured faster, becoming substrates for aminoacylation rather than polyadenylation (Figure 2A). Deregulation of PAP I synthesis changes the ratio of PAP I to the 3′ → 5′ exonucleases resulting in polyadenylation of mature tRNAs and a concomitant decrease in aminoacylation and protein synthesis resulting in cell death (Figure 2B) [33].

It should also be noted that *E. coli* contains a second enzyme, PNPase that can add **polynucleotide tails** onto the 3′ ends of RNA species [35]. PNPase is a highly conserved protein and has been shown to synthesize polynucleotide tails in many other bacterial species [36–38]. However, there is no current evidence to suggest that either pre-tRNAs or mature tRNA species are substrates for the biosynthetic activity of PNPase. Furthermore, the generality of tRNA polyadenylation in prokaryotes may be limited by the number of organisms that contain a true poly(A) polymerase [39]. However, tRNA polyadenylation is not limited to prokaryotes, since polyadenylation of pre-tRNAs has now been observed in a eukaryotic organism [40].

Poly(A) Tail Lengths Vary Significantly between mRNAs and tRNAs

While *E. coli* PAP I primarily adds **homopolymeric poly(A)** tails, PNPase adds mostly poly(A) rich polynucleotide tails containing all four nucleotides [23,34,35,41]. Transcripts with a Rho-independent transcription terminator are usually polyadenylated downstream of the terminator, which serves as a polyadenylation signal for mRNAs in *E. coli* [23,41,42]. Poly(A) tails found on mRNAs in wild-type cells are highly heterogeneous in length ranging from less than 10 to over 50 nts [25,34,43], but can be significantly increased in length to over 125 nts by increasing the intracellular levels of PAP I [34].

Transcripts with Rho-dependent transcription terminators are mostly targeted by PNPase for addition of polynucleotide tails [23,41]. Polynucleotide tails tend to be long, often over 200 nts [25,35,41,44]. The exact reason for why such a variation in tail length or what fraction of transcripts contains longer tails is not clear. Under normal physiological conditions, poly(A) tail length in exponentially growing cells is controlled through a competition between poly(A) synthesis by PAP I and degradation of the poly(A) tails by 3′ → 5′ exonucleases, specifically by PNPase and RNase II [35,45,46]. In addition, the Sm-like protein Hfq, a small RNA-binding protein, also affects the length of poly(A) tails associated with mRNAs *in vivo* [41,47].

In contrast, the poly(A) tails found on tRNA substrates are inherently short (≤10 nts) [18]. In fact, even under conditions where the synthesis of PAP I is significantly increased, the poly(A) tails found on tRNAs are still not longer than 10 nts [18,33], suggesting a significantly reduced processivity of PAP I on tRNA substrates compared with mRNAs. PAP I can add long poly (A) tails to yeast tRNAs *in vitro* [48]. However, the basis for how PAP I discriminates among tRNAs and other RNA species *in vivo* is not clear. It has been reported that the RNA binding protein Hfq plays a significant role in increased processivity of PAP I during mRNA polyadenylation [41,47,49]. However, a comparison of poly(A) tail profiles in *hfq* mutants suggested no effect of Hfq on tRNA polyadenylation [18]. This is probably due to the fact that the poly(A) binding site of Hfq is distinct from its tRNA binding site, which is on its proximal surface [50].

PAP I belongs to the class II group of the nucleotidyltransferase superfamily, which includes bacterial tRNA nucleotidyltransfereases [51,52]. Both enzymes are similar in their amino acid sequences and are involved in template independent addition of nucleotides. However, while PAP I adds poly(A) tails to many different RNA substrates, tRNA nucleotidyltransferases add a maximum of three nucleotides (CCA) to tRNA 3′ ends. Mutational analysis suggests that a set of amino acids that determines the specificity of the CCA-adding enzyme for CTP and ATP are

also conserved in PAP I, but in this case only confer ATP specificity to the enzyme [53]. Thus, it is possible that PAP I retains its intrinsic property to add short poly(A) tails to tRNA substrates *in vivo*. It is also possible that the complex secondary and tertiary structures associated with tRNAs play a role in limiting tRNA poly(A) tail lengths.

Unlike mRNA polyadenylation, the 3′ → 5′ exonucleases PNPase and RNase II, which are involved in limiting poly(A) tail lengths on mRNAs, have no effect on the level and the length of tRNA poly(A) tails [18]. This result is consistent with the fact that both these exoribonucleases require at least a 6–9-nt single-stranded region downstream from the base of a stable stem-loop structure for efficient binding activity [54]. Since the majority of the poly(A) tails associated with tRNAs are shorter than 10 nts, the binding of PNPase and/or RNase II is likely inhibited. Instead, RNase T and RNase PH directly regulate the level and length of tRNA polyadenylation [18]. The mechanism of regulation of tRNA polyadenylation by RNase T and RNase PH is not clear. It is most likely due to rapid processing of immature tRNA 3′ ends to mature 3′ ends, thereby promoting aminoacylation, rather than through the degradation of the poly(A) tails by these enzymes [33]. There is about a tenfold excess of RNase T compared with RNase PH under normal physiological conditions [33]. The number of molecules of both RNase T and RNase PH collectively is higher than the 32–50 molecules of PAP I per cell [41]. Thus, the exoribonucleases have a higher probability of processing a tRNA substrate, allowing it to enter the aminoacylation pathway rather than becoming a substrate for PAP I directed polyadenylation (Figure 2). In fact, increased levels of RNase T and/or RNase PH eliminate the toxicity associated with overexpression of PAP I [33]. It should be noted that, the poly(A) tails reported on several eukaryotic pre-tRNAs are also 1–5 nts in length [40].

Role of Polyadenylation in tRNA Metabolism

The general consensus regarding polyadenylation in prokaryotes is that it serves as a targeting mechanism for the more rapid decay of defective mRNAs. A similar suggestion was made when it was shown that a mutated $tRNA^{Trp}$ was degraded rapidly in a poly(A)-dependent manner [32]. In eukaryotes, the polyadenylated pre-tRNAs are also believed to be part of the surveillance pathway where defective pre-tRNAs are targeted to the Trf4/5-Air1/2-Mtr4 polyadenylation (TRAMP)–exosome pathway [40].

With regard to prokaryotic tRNAs, a more detailed study in *E. coli* involving a variety of tRNAs suggests that under normal physiological conditions only pre-tRNAs are substrates for polyadenylation [18]. However, only a small fraction of the polyadenylated pre-tRNAs are actually degraded [18], possibly via a PNPase-dependent pathway [32], but the bulk of the polyadenylated pre-tRNA species undergo a slow maturation process employing 3′ → 5′ exonucleases that compete with PAP I for the tRNA 3′ ends to generate mature 3′ ends with a CCA trinucleotide. Thus, polyadenylation of appears to slow down the pre-tRNA processing by the 3′ → 5′ exoribonucleases resulting in a potential reduction in functional tRNA levels [18]. The aminoacylation of mature tRNAs most likely prevents their polyadenylation (Figure 2A) (see Outstanding Questions).

Consequently, the absence of 3′ → 5′ processing exonucleases significantly increases the extent of pre-tRNA polyadenylation [4,18,55,56]. Since RNase T is both the most abundant and effective 3′-end processing enzyme, it strongly prevents polyadenylation of mature tRNAs (Figure 2A) [33]. Increasing PAP I levels above RNase T levels leads to widespread polyadenylation of mature tRNAs resulting in the reduction of aminoacylation, inhibition of protein synthesis, and ultimately cell death (Figure 2B) [33]. Accordingly, the intracellular level of PAP I plays a balancing role in maintaining normal levels of functional tRNAs *in vivo*.

While polyadenylation of mRNAs and defective RNAs is important for their rapid degradation as part of general quality control, PAP I levels are strictly regulated in multiple ways in order to avoid the polyadenylation of tRNAs and to maintain a normal level of functional tRNAs. PAP I expression has been shown to be limited by an inefficient start codon (AUU) and a poor ribosome binding site [57]. In addition, the presence of at least four internal Shine–Dalgarno-like elements within the coding sequence also limits protein synthesis by translational pausing [58].

Surprisingly, the three proline tRNAs are processed in a unique way and avoid polyadenylation at their 3′ ends. Previous studies have shown that the initial endonucleolytic processing of tRNA operons by RNase E leaves pre-tRNAs with 1–3 extra nucleotides downstream of the CCA determinant, which are subsequently removed by the 3′ → 5′ exoribonucleases [6,8,12]. The unprocessed nucleotides prevent aminoacylation, thereby providing substrates for polyadenylation by PAP I. However, a detailed analysis of the processing of the three proline tRNAs has clearly shown that RNase E cleaves immediately downstream of the CCA determinant for all three proline species, thereby avoiding polyadenylation [19]. A similar type of 3′-end endonucleolytic processing is also predicted for the four initiator methionine tRNAs, which are also not substrates for polyadenylation [18].

Concluding Remarks and Future Perspectives

The role of polyadenylation in tRNA metabolism is still emerging particularly since the full consequences of tRNA polyadenylation by PAP I and the various elements regulating this phenomenon are still not completely understood (see Outstanding Questions). The distribution of bacterial PAP is limited to β, γ, and δ subdivisions of proteobacteria and in some Chlamydiales and Spirochaetales [52]. It is absent in α and ε proteobacterial subdivisions including all Gram-positive bacteria [52]. Thus, the limited occurrence of a true PAP I type enzyme in most bacteria raises the question as to why *E. coli* has the enzyme. Based on the demonstration that polyadenylation is involved in regulating functional tRNA levels in *E. coli*, it is possible that the absence of PAP I is related to avoiding the inherent toxicity associated with the polyadenylation of mature tRNAs. *E. coli* has dealt with this problem by significantly downregulating the expression of PAP I, through the absence of a good ribosome binding site and the utilization of a non-optimal translation start codon, such that there are only 32–50 molecules per cell [41]. Since increased expression of PAP I in wild-type cells leads to significant toxicity [34], it is possible that the presence of the enzyme presents a selective disadvantage to organisms where expression has not been downregulated. Furthermore, it has been argued that many organisms lack a true PAP enzyme because they contain PNPase, which can carry out poly(A) additions [51]. However, it is not clear that this is a reasonable explanation, since PNPase adds long polynucleotide tails as opposed to the both short and long poly(A) tails that are added by PAP I to RNA substrates. More importantly, there is no current evidence that pre-tRNAs are substrates for the biosynthetic activity of PNPase.

Outstanding Questions

Is the abundance of polyadenylated pre-tRNAs/tRNAs and/or the length of their poly(A) tails regulated in response to growth conditions, growth phase, or stress?

Why are mature tRNA 3′ ends poorly polyadenylated compared with immature 3′ ends? Is it because of low PAP I levels or immediate aminoacylation?

The three proline and four initiator met tRNAs avoid polyadenylation at their 3′ end by employing an RNase E endonucleolytic cleavage immediately downstream of the CCA determinant. What distinguishes these tRNAs from the other 79 tRNAs found in *E. coli*?

Why is RNase P essential in *E. coli*? Is it because of its role in the 5′-end maturation of pre-tRNAs, or the failure to separate the pre-tRNAs found in RNase P-dependent tRNA operons, or the rapid degradation of 5′ immature tRNAs, which are subject to polyadenylation?

The maturation pathways of the 14 tRNAs embedded in ribosomal operons (Table 1) are still unknown. Do they follow one of the above-described pathways (Figure 1) or other independent pathways?

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