Published online 14 December 2012 *Nucleic Acids Research, 2013, Vol. 41, No. 3* ***1757–1766***
doi:10.1093/nar/gks1280

Deregulation of poly(A) polymerase I in *Escherichia coli* inhibits protein synthesis and leads to cell death

Bijoy K. Mohanty and Sidney R. Kushner*

Department of Genetics, University of Georgia, Athens, GA 30602, USA

Received October 2, 2012; Revised November 7, 2012; Accepted November 8, 2012

ABSTRACT

Polyadenylation plays important roles in RNA metabolism in both prokaryotes and eukaryotes. Surprisingly, deregulation of polyadenylation by poly(A) polymerase I (PAP I) in *Escherichia coli* leads to toxicity and cell death. We show here that mature tRNAs, which are normally not substrates for PAP I in wild-type cells, are rapidly polyadenylated as PAP I levels increase, leading to dramatic reductions in the fraction of aminoacylated tRNAs, cessation of protein synthesis and cell death. The toxicity associated with PAP I is exacerbated by the absence of either RNase T and/or RNase PH, the two major 3′ → 5′ exonucleases involved in the final step of tRNA 3′-end maturation, confirming their role in the regulation of tRNA polyadenylation. Furthermore, our data demonstrate that regulation of PAP I is critical not for preventing the decay of mRNAs, but rather for maintaining normal levels of functional tRNAs and protein synthesis in *E. coli*, a function for polyadenylation that has not been observed previously in any organism.

INTRODUCTION

Polyadenylation of RNA, particularly mRNAs, is widespread in all forms of life and is carried out by both canonical and non-canonical poly(A) polymerases (PAPs) (1–4). In *Escherichia coli* the non-canonical poly(A) polymerase I (PAP I), encoded by the *pcnB* gene (5), serves as the primary polyadenylating enzyme (6,7). In addition, polynucleotide phosphorylase (PNPase) encoded by the *pnp* gene, a major 3′→5′ exoribonuclease, adds 3′ heteropolymeric tails and accounts for ∼10% of the total post-transcriptionally added 3′ tails in wild-type *E. coli* (7,8). Interestingly, many features of prokaryotic polyadenylation are distinct from the well-studied eukaryotic polyadenylation pathways associated with canonical PAPs. For example, addition of poly(A) tails increases the stability of transcripts in eukaryotes (9,10), whereas it promotes instability in prokaryotes (6,11,12). More importantly, the long poly(A) tails associated with eukaryotic mRNAs improve translation efficiency (13,14). In contrast, polyadenylation of bacterial mRNAs has not been shown to have any effect on their translation.

Surprisingly, although transcripts from majority of the open reading frames in wild-type *E. coli* are polyadenylated to some extent in exponentially growing cells (7), it has been estimated, using oligo(dT) columns, that only ∼2% of total RNA is polyadenylated at any given time (15). In fact, the low *in vivo* level of PAP I protein (16) seems to be the rate limiting factor in controlling *in vivo* poly(A) levels, as increased expression of PAP I raised the level of all polyadenylated transcripts tested (6,7). The *pcnB* gene in *E. coli* has a moderately strong promoter, but translation is significantly limited by a non-canonical initiation codon (UUG) and a poor ribosome binding site (6,17). In fact, it has been estimated that there are only 30–50 molecules of PAP I per cell (16) compared with >350 000 total 3′ RNA termini (18).

It was thus of considerable interest that although deletion of the *pcnB* gene has only a minimal effect on growth rate in rich medium (19), increased expression of PAP I led to slower growth and cell death (5,6). We subsequently determined that survivors obtained from an IPTG-induced *pcnB* expression plasmid under the control of the *lac* promoter (6) contained a single nucleotide change (C → A) in the −35 region of the *lac* promoter that effectively blocked transcription of the *pcnB* gene. Because PAP I deregulation also led to reduced half-lives of some mRNAs (6), we speculated that increased PAP I levels led to significant destabilization of mRNA(s) that encoded proteins essential for cell viability.

To test this hypothesis, we simultaneously overexpressed either PNPase or RNase II, the primary two 3′ → 5′ exonucleases known to degrade poly(A) tails *in vivo* (20), along with PAP I. Overexpression of PNPase did reduce the total *in vivo* poly(A) level and average length of the poly(A) tails, but did not improve

*To whom correspondence should be addressed. Tel: +1 706 542 8000; Fax: +1 706 542 3910; Email: skushner@uga.edu

the growth rate or cell viability (20). In contrast, overexpression of RNase II led to a faster growth rate, but this result arose from low PAP I levels directly related to a significant reduction in the copy number of the plasmid carrying the *pcnB* gene (20). More importantly, a macroarray study, using total RNA derived from cells containing more than a 100-fold increase in PAP I activity (6), did not show any significant changes in the steady-state levels of transcript(s) that might be essential for cell viability (7). However, the macroarray used only probed for transcripts from open reading frames (7), thereby excluding non-coding RNAs, such as rRNAs, sRNAs and tRNAs, which can serve as substrates for PAP I under certain circumstances (6,19,21–24).

In *E. coli*, the majority of tRNA 3′-ends are primarily matured by either RNase T (encoded by *rnt*) or RNase PH (encoded by *rph*) (19). Studies have shown that partially processed tRNAs (pre-tRNAs) are polyadenylated by PAP I in the absence of RNase T and/or RNase PH (25–27), leading to a large accumulation of species that are not charged by aminoacyl tRNA synthetases and significantly reduced growth rates (19). More importantly, however, no poly(A) tails have been previously detected on mature tRNAs (those ending with a CCA at the 3′ terminus) in wild-type cells (6,22,28,29). Thus, it was proposed that pre-tRNAs are protected from polyadenylation owing to rapid processing by the more abundant RNase T and RNase PH enzymes and their immediate aminoacylation (19). Since addition of even a single nucleotide to the mature 3′-end would generate a tRNA that could not be aminoacylated, we suspected that changes in the *in vivo* ratios of PAP I, RNase T and RNase PH would result in the polyadenylation of mature tRNAs, leading to reduced growth rates and cell death.

We show here that deregulation of PAP I, in fact, leads to the rapid polyadenylation of mature tRNAs, particularly $tRNA^{His}$, $tRNA^{Cys}$ and $tRNA^{Metm}$, resulting in dramatic reductions in functional tRNA levels and protein synthesis along with concomitant growth retardation and cell death. These results demonstrate a new function for polyadenylation that has not been previously seen in any biological organism. Furthermore, deregulation of PAP I in the absence of either RNase PH or RNase T or both enzymes significantly increased the levels of non-functional tRNAs and the loss of cell viability compared with a wild-type control strain. In contrast, overexpression of either RNase T or RNase PH suppressed the toxicity associated with increased levels of polyadenylation. The data presented here strongly suggest that the lethality associated with the deregulation of PAP I in *E. coli* is not related to RNA quality control, but is due to a direct role in depleting the optimal levels of functional tRNAs.

MATERIALS AND METHODS

Bacterial strains and plasmids

All strains used in this study were derived from MG1693 (*rph-1 thyA715*) (30). SK10153 (*thyA715*) (19) and SK9124 [*rph-1 thyA715*/pBMK11($pcnB^+$/Cm^R)] (6) have been described previously. SK10574 [*Δrnt rph-1 thyA715*/pBMK11($pcnB^+$/Cm^R)], SK10620 [*thyA715*/pBMK11($pcnB^+$/Cm^R)] and SK10621 [*Δrnt thyA715* /pBMK11($pcnB^+$/Cm^R)] were constructed by transforming pBMK11 ($pcnB^+$/Cm^R) (6) into SK10148 (*Δrnt rph-1 thyA715*) (31), SK10153 (*thyA715*) and SK10592 (*Δrnt thyA715*) (19), respectively. SK10647 [*thyA715*/pBMK11($pcnB^+$/Cm^R)/pBMK58 (rnt^+/Ap^R)] and SK10667 [*thyA715*/pBMK11($pcnB^+$/Cm^R)/pBMK61 (rph^+/Ap^R)] were constructed by transforming pBMK58 (rnt^+/Ap^R) (19) and pBMK61(rph^+/Ap^R), respectively, into SK10620 [*thyA715*/pBMK11($pcnB^+$/Cm^R)].

Plasmids pBMK11 ($pcnB^+$/Cm^R) (6) and pBMK58 (rnt^+/Ap^R) (19) have been described previously. Plasmid pBMK61(rph^+/Ap^R) was constructed by cloning the *rph* coding sequence containing its own promoter into a Ap^R resistance derivative of the 6-8 copy vector pWSK29 (32) using the *Pst*I - *Bam*HI sites. A polymerase chain reaction fragment containing the *rph* coding sequence was amplified using primer pairs RPH-PST (247 nt upstream of ATG start codon) and RPH-1291 (347 nt downstream of TAG translation stop codon) using Phusion™ high-fidelity DNA polymerase (NEB).

Northern and western blotting analysis

Total RNA isolation, northern and western blot analysis (PAP I, RNase T and RNase PH) were carried out as described previously (19) with following modifications. For PAP I induction, IPTG (350 μmol) was added to cultures at 50 Klett units (No. 42 green filter) above background. RNase T and RNase PH were detected using polyclonal antibodies raised against RNase T- and RNase PH-specific peptides by GenScript USA Inc, NJ. The membrane was probed simultaneously with both RNase T (1:10 000) and RNase PH (1:5000) antibodies using Pierce® ECL western blotting substrate.

Determination of aminoacylation levels of tRNAs

The percentage of tRNA aminoacylation was determined as described previously (19).

Probe selection and quantification of data

Specific oligonucleotide probes used to detect all tRNAs and quantification of data have been described (19).

Cloning and sequencing of tRNA ends

The 5′- and 3′-ends of tRNAs were identified using methods described previously (28).

RESULTS

Deregulation of PAP I leads to rapid polyadenylation of tRNAs

Because RNase T and RNase PH prevent the polyadenylation of both mature and precursor tRNAs by the tightly regulated PAP I in wild-type *E. coli* (19), we hypothesized that deregulation of PAP I would overwhelm this mechanism and lead to the polyadenylation of all tRNAs, including the mature species. To test this idea,

total RNA was isolated from various strains at specific time intervals after PAP I synthesis was deregulated by IPTG induction of pBMK11(*pcnB*$^{+}$/Cm^{r}) (6) and was analysed by northern analysis for specific tRNAs. The mature species of tRNA^{His}, tRNA^{Cys} and tRNA^{Metm} showed no sign of polyadenylation within 15 min after IPTG induction in the wild-type control (SK10153) or a wild-type strain carrying pBMK11 (SK10620, Figure 1, lanes 1–4). However, altered tRNA mobilities, associated with the addition of short poly(A) tails, were observed after 45 min in the wild-type/pBMK11 (SK10620) strain (data not shown). In contrast, altered tRNA mobilities were observed by 15 min in the *rph-1*/pBMK11 strain (SK9124) (Figure 1, lane 9) and by 4 min in the *Δrnt*/pBMK11 strain (SK10621) (Figure 1, lanes 12-14). By 15 min, the majority of the mature tRNAs in the *Δrnt*/pBMK11 strain were higher-molecular-weight species (Figure 1, lane 14). However, tRNA^{Pro} only showed minor polyadenylation in the *Δrnt*/pBMK11 strain even after 15 min of PAP I induction (Figure 1, lane 14), a result consistent with our previous observation that tRNA^{Pro} was resistant to polyadenylation (19).

To determine where the poly(A) tails were being added to the tRNAs, we analysed the 3′-ends of the *hisR* and *cysT* transcripts after PAP I induction (Figure 2). Because these tRNA genes are present in single copy, any significant change in their functional levels would immediately affect growth and cell viability. The RNA sample isolated from an *rph-1*/pBMK11 strain (SK9124) 15 min after PAP I induction (Figure 1, lane 9) was used for this experiment, as this genetic background showed an intermediate level of polyadenylation compared with the *Δrnt*/pBMK11 strain (SK10621) and the wild-type/pBMK11 control (Figure 1, lanes 4, 14). The 5′-3′ junctions of ligated *hisR* and *cysT* transcripts were cloned and sequenced as previously described (28). The 3′-ends of all of the cloned *hisR* and *cysT* transcripts had poly(A) tails ranging from 1 to 12 nt in length (Figure 2). As predicted, ~45% (11/24 clones for each tRNA) of the transcripts had poly(A) tails added immediately after the mature CCA terminus. This result was in stark contrast to an *rph-1* control strain (no PAP I induction) where no polyadenylated mature tRNAs were observed (19,28).

Functional tRNA levels and protein synthesis are significantly reduced after PAP I induction

Irrespective of the genetic system, the acceptor stem of the tRNA must contain a CCA trinucleotide as a prerequisite for tRNA aminoacylation (33), as the activated amino acid is attached to the terminal A residue. Any additional nucleotide(s) downstream of the CCA, such as A residues added by PAP I (Figure 2), would not only block the amino acid attachment site but would also inhibit the interaction between the tRNA and 23S rRNA through the CCA in the ribosomal P site (34,35). Accordingly, we measured the *in vivo* aminoacylation levels of selected tRNAs in various genetic backgrounds using the method of Varshney *et al.* (36). As expected, there were significant reductions in the levels of aminoacylated tRNAs in both the wild-type/pBMK11 and *rph-1*/pBMK11 strains after PAP I induction (Figure 3 and Table 1). However, the reduction in the level of aminoacylated tRNAs was most dramatic (*e.g.* 30–34-fold reductions in tRNA^{Cys} and tRNA^{His}, respectively) in the *Δrnt* and *Δrnt rph-1* genetic backgrounds (Figure 3 and Table 1). No significant

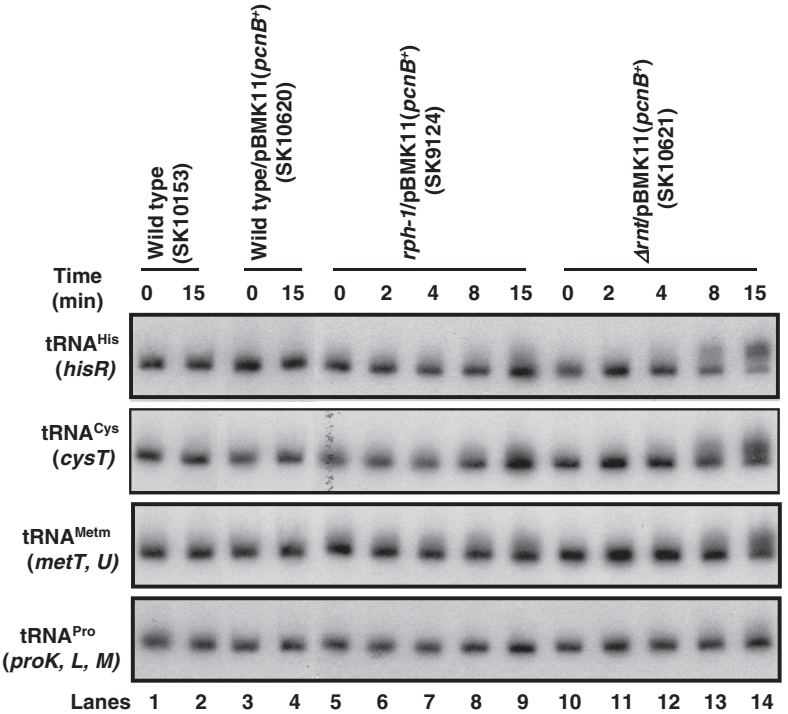


Figure 1. Time course of tRNA polyadenylation after deregulation of PAP I synthesis. Strains were grown to Klett 50 and induced with IPTG (350 μmol). Total RNA (12 μg/lane) isolated at indicated time was analysed by northern analysis.

change in the aminoacylation level of $tRNA^{Pro}$ was observed in any genetic background (Figure 3 and Table 1), again consistent with our previous finding that this tRNA was not a substrate for PAP I (19).

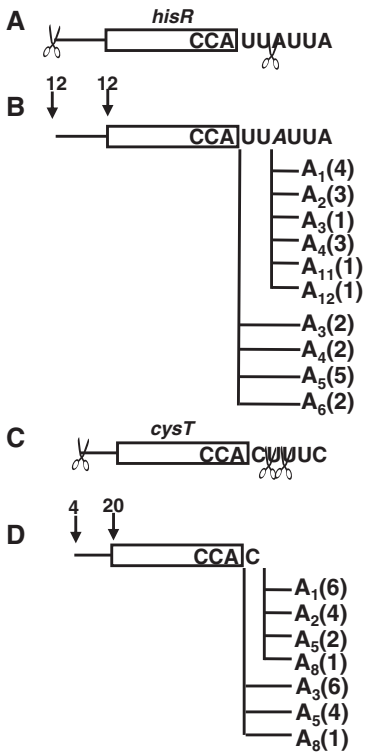


Figure 2. Characterization of the 5′ and 3′ termini of $tRNA^{His}$ (A-B) and $tRNA^{Cys}$ (C-D) after PAP I induction in the *rph-1* (SK9124) strain using reverse transcriptase polymerase chain reaction cloning of 5′-3′ self-ligated transcripts. Schematic presentation of (A) *hisR* and (C) *cysT* coding sequences is shown. Scissors at the 5′ upstream end and in the 3′ trailer sequences downstream of the CCA terminus represent endonucleolytic cleavages by RNase E (28). The sequenced 5′-ends are shown as solid inverted arrows and the 3′-ends are as ladders with untemplated As. In some cases, the sources of A cannot be determined (italicized *A*). The numbers directly above each arrow or besides untemplated As represent the number of clones identified with these respective 5′- or 3′-ends.

Because a drop in the fraction of aminoacylated tRNAs would be expected to inhibit protein synthesis, we directly measured the incorporation of ^{35}S-labeled methionine and cysteine to confirm this idea. As shown in Figure 4A, deregulation of PAP I in SK10620 (wild-type/pBMK11) led to a small reduction in incorporation after 30 min of IPTG induction. As suggested by the small change in charged tRNAs between the wild-type and *rph*-1 strains (Table 1), the reduction in incorporation was only slightly

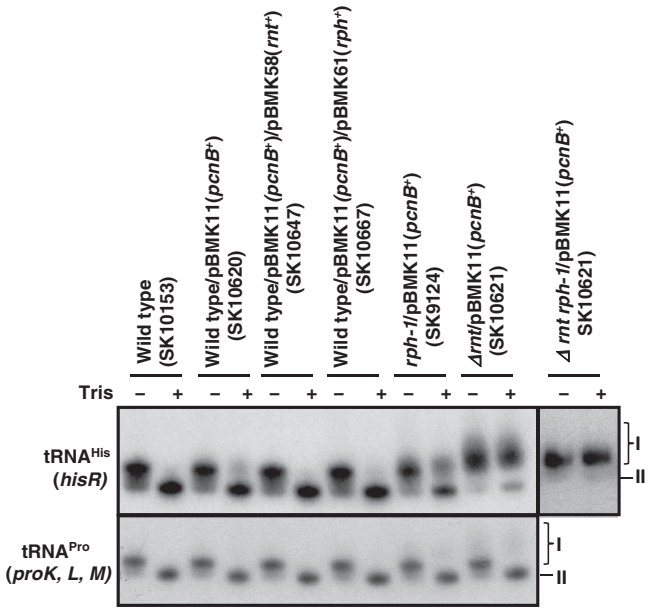


Figure 3. Representative northern blots showing the steady-state aminoacylation levels of $tRNA^{His}$ and $tRNA^{Pro}$. Total RNA isolated from various strains was untreated (−) or treated (+) with 0.5 M Tris (pH 9) to chemically deacylate tRNAs and was separated using acid urea polyacrylamide gel as described in Materials and Methods. The position marked with I indicated either charged and/or high-molecular-weight non-functional tRNAs [with poly(A) tails]. The position marked with II indicated uncharged form of tRNA. The net extent of aminoacylation for band I was calculated as percentage of total counts (bands I + II) minus the percentage of high-molecular-weight species after Tris treatment (19). The aminoacylation levels of all tRNAs studied are reported in Table 1.

Table 1. Aminoacylated tRNA levels in various strains after PAP I induction

tRNA isotype/genes	Percentage of aminoacylated tRNAs						
	Wild-type (SK10153)	Wild-type/ pBMK11 (SK10620)	*rph-1*/pBMK11 (SK9124)	*Δrnt*/pBMK11 (SK10621)[a]	*Δrnt rph-1*/ pBMK11 (SK10574)[a]	Wild-type/ pBMK11/pBMK58 (SK10647)	Wild-type/ pBMK11/pBMK61 (SK10667)
His/*hisR*	78 ± 2	59 ± 4	47 ± 3	8 ± 1	2 ± 0.5	76 ± 2	76 ± 2
Cys/*cysT*	60 ± 2	54 ± 3	50 ± 3	10 ± 1	2 ± 0.5	57 ± 2	56 ± 2
Leu5/*leuX*	92 ± 2	86 ± 2	81 ± 2	47 ± 2	20 ± 2	92 ± 2	93 ± 2
Metm/*metT, U*	84 ± 2	78 ± 2	62 ± 3	5 ± 1	ND	83 ± 2	85 ± 1
Ala1B/*alaT, U, V*	80	76	28	20	ND	85	83
Phe/*pheU, V*	83	77	66	35	ND	83	82
Arg2/*argQ, V, Y, Z*	72 ± 3	69 ± 3	60 ± 2	30 ± 4	ND	80 ± 2	92 ± 2
Pro/*proK, L, M*	77 ± 1	77 ± 1	78 ± 1	75 ± 1	ND	78 ± 2	78 ± 2

Data with no standard deviations indicate that the experiment was done once. ND: not determined.
[a]SK10621and SK10574 were induced for 30 min before sampling, as these strains lose cell viability more quickly than the other strains (Figure 1). All other strains were induced for 60 min before sampling. pBMK11, $pcnB^+$; pBMK58, rnt^+; pBMK61, rph^+.

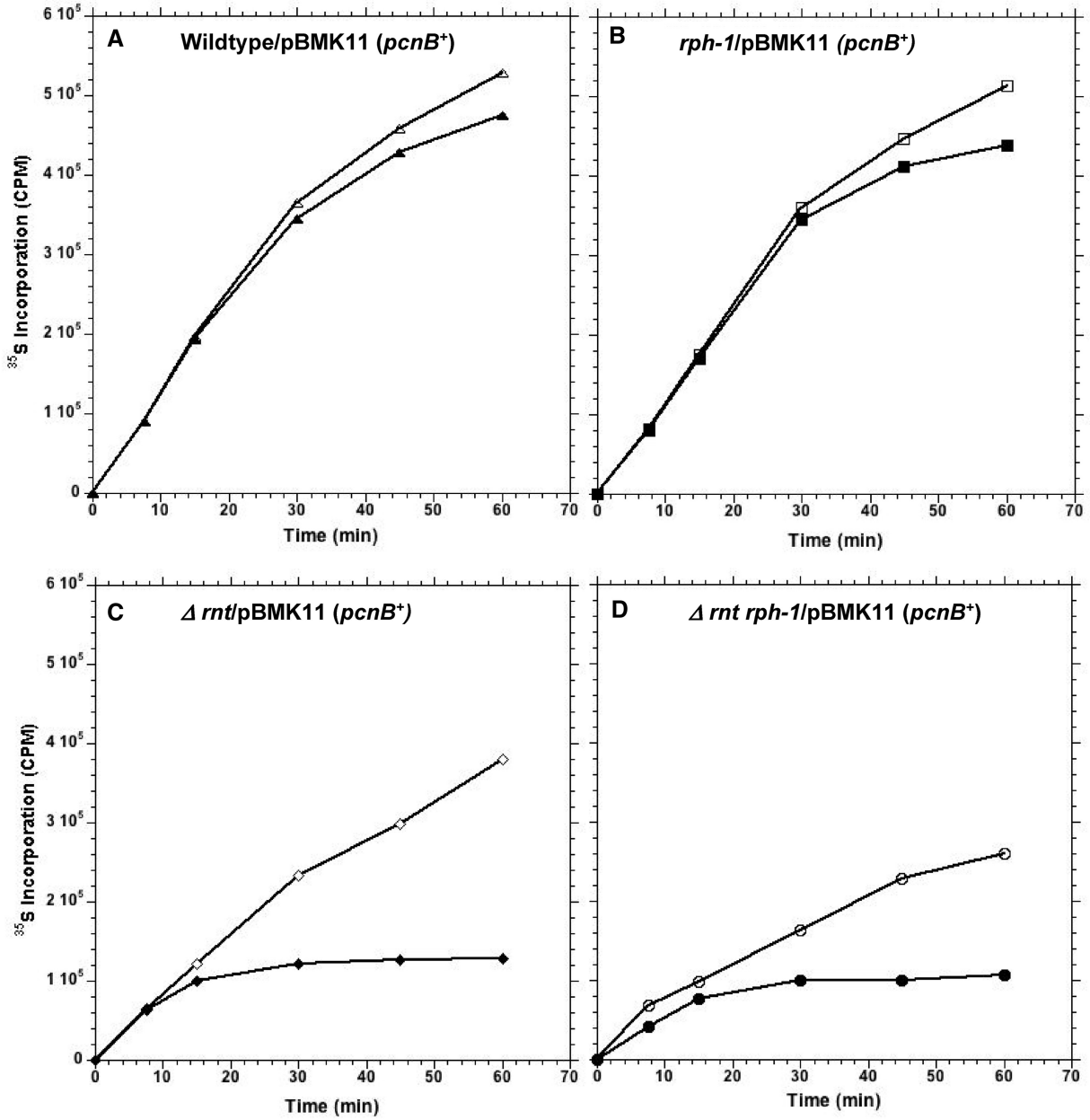


Figure 4. Effect of deregulation of PAP I expression on protein synthesis in (**A**) SK10620, (**B**) SK9124, (**C**) SK10621 and (**D**) SK10574 genetic backgrounds. ^{35}S-methionine/cysteine protein labeling mix (EasyTag^{TM} EXPRE^{35}S^{35}S Protein labeling mix containing [^{35}S]-methionine and L-[^{35}S]-cysteine, 11 mCi/ml, NEN Radiochemicals, Perkin Elmer®) was added either without (open symbols) or with (closed symbols) IPTG (350 µmol) at 50 Klett units above background (time point 0). At various times, samples (500 µl) were removed and added to 4 ml of cold trichloroacetic acid (10%) and stored on ice for 60 min. After filtering through GF/C filter papers (Whatman®), the acid-precipitable ^{35}S-material was counted in a Beckman LS6000IC scintillation counter.

more pronounced in the isogenic *rph-1*/pBMK11 strain (SK9124, Figure 4B). In contrast, in the *Δrnt*/pBMK11 strain (SK10621), the rapid polyadenylation of tRNAs (Figure 1) as well as large drops in the levels of charged tRNAs, particularly tRNA^{His}, tRNA^{Cys} and tRNA^{Metm} (Table 1), directly correlated with a complete cessation of protein synthesis within 30 min after induction (Figure 4C). Protein synthesis stopped by 15 min after induction in the *Δrnt rph-1*/pBMK11 strain (SK10574) (Figure 5D), as predicted by the further drop in the level of charged tRNAs, particularly the almost complete absence of functional tRNA^{His}, tRNA^{Cys} and tRNA^{Metm} (Table 1).

The toxicity associated with increased levels of PAP I is directly related to the levels of RNase PH and/or RNase T

Based on the data presented previously, we predicted that the toxicity previously observed with the deregulation of PAP I synthesis in an *rph-1* mutant of *E. coli* (6) would be significantly reduced in an *rph*$^{+}$ derivative. In fact, the

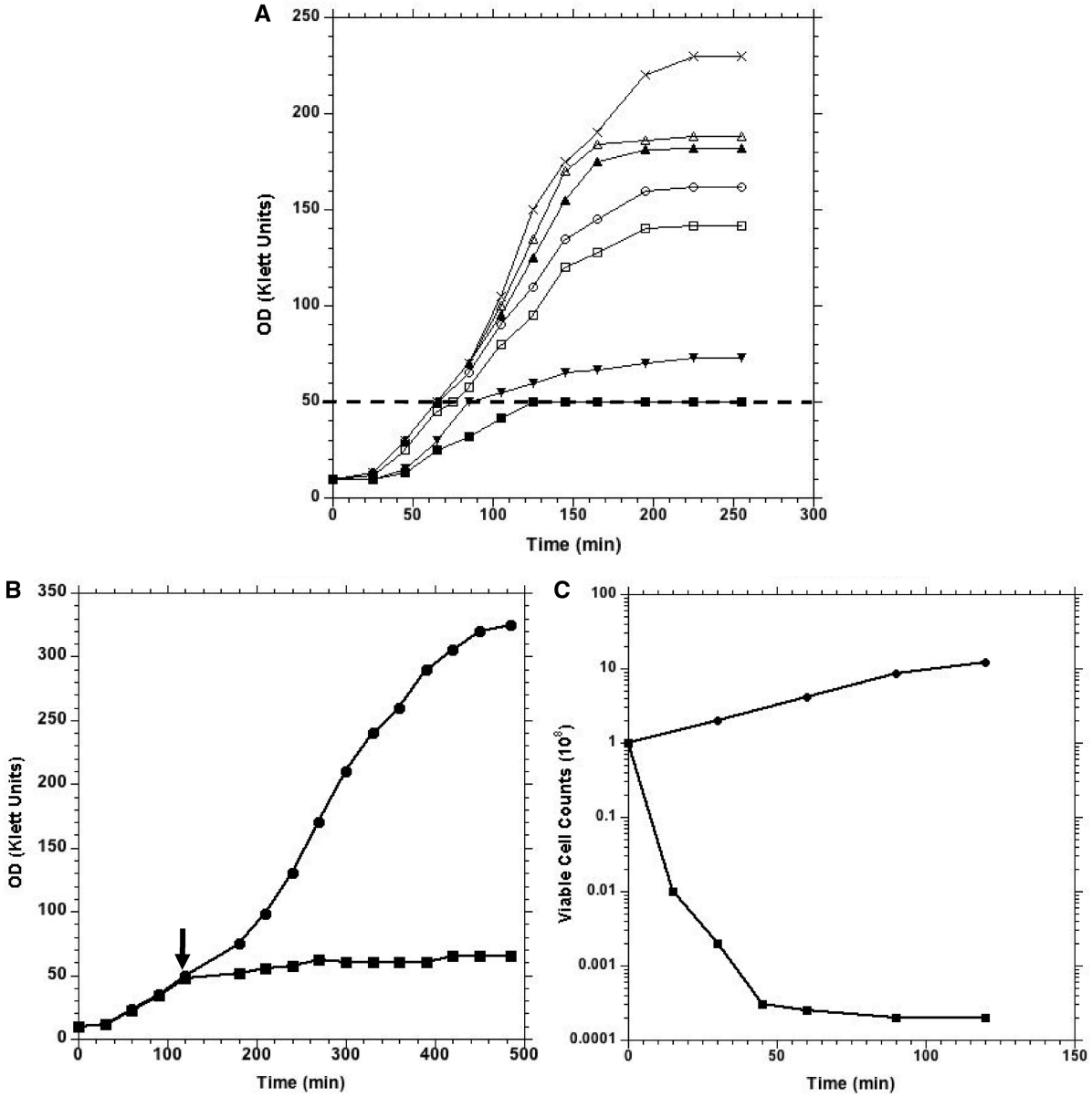


Figure 5. Growth curves and viable cell counts of various strains. (**A**) Comparison of growth rates of strains after IPTG (350 μmol) addition at 50 Klett units (broken parallel line). The optical densities (OD) of all the strains without dilution were measured in Klett units and plotted against time. SK10153 (wild-type, X), SK10620 (wild-type/pBMK11, white circle), SK9124 (*rph-1*/pBMK11, white square), SK10621 (*Δrnt*/pBMK11, black down-pointing triangle), SK10667 (wild-type/pBMK11/pBMK61, black up-pointing triangle), SK10647 (wild-type/pBMK11/pBMK58, Δ) and SK10574 (*Δrnt rph-1*/pBMK11, black square) (**B**) Growth rate and (**C**) cell viability of SK10574 [*Δrnt rph-1*/pBMK11(Cm^R/*pcnB*$^+$)] after PAP I induction by IPTG at 50 Klett units (arrow in B and 0-min time point in C). Cells were diluted with pre-warmed LB (black circle) or LB + IPTG (black square) to maintain the exponential growth.

growth rate of the wild-type/pBMK11 strain (SK10620) was faster compared with the *rph-1*/pBMK11 strain (SK9124) after PAP I induction (Figure 5A). Conversely, because the polyadenylation level of tRNA precursors as well as mature tRNAs increased significantly in a *Δrnt* strain compared with an *rph-1* strain (Figure 1), along with dramatic reductions in aminoacylated tRNA levels and protein synthesis (Table 1 and Figure 3), we expected and observed that deregulation of PAP I synthesis had a much more dramatic effect on growth rate in a *Δrnt*/pBMK11 strain (SK10621) compared with either the wild-type/pBMK11 (SK10620) or *rph-1*/pBMK11 (SK9124) strains (Figure 5A). Furthermore, induction of PAP I in a *Δrnt rph-1* double mutant (SK10574, *Δrnt rph-1*/pBMK11) led to complete growth arrest within minutes of IPTG addition (Figure 5A and B) and a rapid drop in viable cells such that after 60 min of PAP I induction, there was a >99.9% loss in cell viability (Figure 5C).

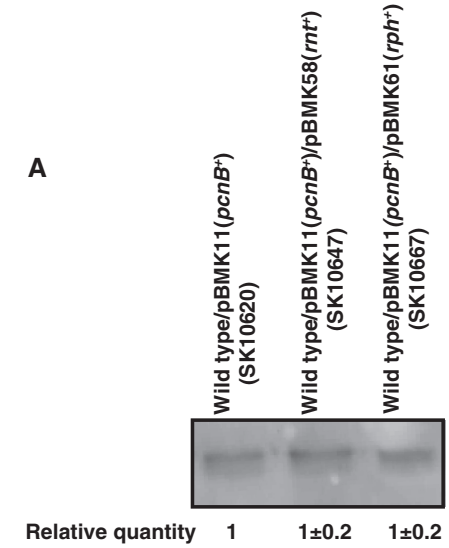
Alternatively, we constitutively overexpressed either RNase T from pBMK58 (rnt^+/Ap^r) (19) in SK10647 (wild-type/pBMK11/pBMK58) or RNase PH from

pBMK61 (rph^+/Ap^r) in SK10667 (wild-type/pBMK11/pBMK61) simultaneously with PAP I induction. Both pBMK58 and pBMK61 are pSC101-based plasmids (six to eight copies/cell) expressing ~4–6-fold more RNase T and RNase PH, respectively, compared with a wild-type control. Both strains had growth rates that were comparable with the wild-type control strain (SK10153) up to 100 min after PAP I induction (Figure 5A). Subsequently, the strains entered stationary phase at lower cell densities than the wild-type control, but at significantly higher levels than were observed with the *rph-1*/pBMK11 (SK9124) or *Δrnt*/pBMK11 (SK10621) strains (Figure 5A).

However, it has been previously shown that simultaneous overexpression of PAP I and RNase II partially suppressed the toxicity associated with increased PAP I levels, but this observation resulted from a significant reduction in PAP I protein levels related to a decrease in the copy number of pBMK11 ($pcnB^+$/Cm^R) (20). To find out if overexpression of RNase PH and RNase T also led to reduction in PAP I levels, we carried out western blot analysis to compare the PAP I levels in SK10620 (wild-type/pBMK11), SK10647 (wild-type/pBMK11/pBMK58) and SK10667 (wild-type/pBMK11/pBMK61) after 15 min of IPTG induction. The PAP I levels were identical in all the strains tested (Figure 6A). More importantly, the simultaneous overexpression of either RNase PH (SK10667) or RNase T (SK10647) along with PAP I restored aminoacylation to wild-type levels (Table 1 and Figure 3).

Significant differences in the *in vivo* levels of RNase T and RNase PH help explain the phenotypic differences between *Δrnt* and *rph-1* mutants

The significantly higher level of short poly(A) tails (<10 nt) and observed slower growth rate in an *Δrnt* mutant compared with an *rph-1* strain indicated that RNase T was more effective in controlling polyadenylation of tRNA precursors compared with RNase PH (19). These observations were also consistent with the higher level of toxicity observed in the *Δrnt* mutant on deregulation of PAP I synthesis (Figure 5). One possible explanation of these results was that there was more RNase T than RNase PH in the cell. In fact, western blot analysis showed that the level of RNase T to be ~10-fold higher compared with RNase PH in the wild-type cells (Figure 6B).Interestingly, unlike the compensatory changes in PNPase and RNase II levels observed in *Δrnb* and *pnp-7* strains, respectively, compared with a wild-type control (37), inactivation of either RNase T or RNase PH did not lead to any changes in the amount of the other enzyme (Figure 6B).



	Relative quantity		
RNase PH	1	-	1.3 ± 0.2
RNase T	10± 2	10± 2	-

Figure 6. Western blot analysis of PAP I (**A**), RNase PH (B) and RNase T (**B**) proteins in various strains. The PAP I level in SK10620 and the RNase PH level in SK10153 were set at 1. The quantitative comparison of both proteins is based on identical relative sensitivities of the polyclonal antibodies to their respective purified MAP peptides at various dilutions using enzyme-linked immunosorbent assay (data not shown). Relative quantity represents the average of three independent determinations.

DISCUSSION

The data presented here demonstrate for the first time that deregulation of PAP I in *E. coli* is bacteriocidal (Figure 5) as a direct consequence of the inhibition of protein synthesis (Figure 4), which arises from the inactivation of mature tRNAs through their polyadenylation (Figures 1–3 and Table 1). Thus, although the absence of PAP I only leads to a small growth defect, increases in PAP I protein above normal physiological levels result in rapid polyadenylation of mature tRNAs (Figures 1 and 2). Because mature tRNAs are not polyadenylated in wild-type cells (19), our results demonstrate that *in vivo* PAP I levels are tightly regulated to protect the integrity of mature tRNAs. Taken together these data demonstrate a new function for polyadenylation that has not been previously observed in any living organism.

The exact reason why mature tRNAs become rapidly polyadenylated when the enzyme is deregulated is not clear. However, because PAP I can add poly(A) tails to any RNA substrate *in vitro* (38), it may not be surprising that tRNAs, being the major fraction of the total intracellular RNA pool, are immediately targeted for polyadenylation as PAP I levels increase (Figures 1 and 2). Alternatively, when overproduced, PAP I being an ortholog of the CCA-adding enzyme (tRNA

nucleotidyltransferase), which strictly uses tRNAs as substrates, may prefer to target tRNAs, rapidly adding short poly(A) tails (39). Consequently, the polyadenylated tRNAs are not charged (Figure 3) by their respective aminoacyl tRNA synthetases, leading to reduced protein synthesis (Figure 4). This conclusion is supported by the slower growth rate in the wild-type strain after deregulation of PAP I, which is exacerbated in *rph-1* and *Δrnt* single mutants (Figure 5A). In the absence of both RNase T and RNase PH, protein synthesis stopped almost immediately after PAP I induction (Figure 4D) and >99.99% of the cells died within 60 min (Figure 5C).

It has previously been shown that RNase PH and RNase T, the two major tRNA maturation exonucleases, play significant roles in generating mature CCA termini from pre-tRNAs, which are a prerequisite for aminoacylation, (26,33). In addition, these two ribonucleases also protect the mature CCA ends from polyadenylation by PAP I (19). Thus, the significant decrease in aminoacylated tRNAs in the absence of RNase PH and/or RNase T is consistent with rapid conversion of both mature and pre-tRNAs to non-functional tRNAs by post-transcriptional addition of short poly(A) tails (Figure 2). Furthermore, the rate and level of tRNA polyadenylation is directly dependent on the level of these two ribonucleases. Accordingly, the loss of the more abundant RNase T (Figure 6B) results in a much faster and higher level of tRNA polyadenylation compared with the inactivation of RNase PH (Figure 1).

Conversely, co-overexpression of either RNase T or RNase PH (increases of ~4–6-fold) partially suppressed the toxicity associated with the deregulation of PAP I synthesis (Figure 5A). Thus, our data support a hierarchy in the final 3′-end maturation of tRNAs where RNase T, the more abundant ribonuclease (Figure 6), has a much greater impact than RNase PH (Figure 1 and 5 and Table 1). The data are also in agreement with previous findings that RNase T is more effective in preventing polyadenylation by PAP I (19) and that the substrate specificity of this enzyme promotes accurate tRNA processing (40,41). Moreover, the rapid cessation of cell growth in the *Δrnt rph-1* double mutant (Figure 5B), along with the dramatic reduction in cell viability (Figure 5C), indicates that the remaining 3′→ 5′ exonucleases involved in tRNA processing (RNase D, RNase BN and RNase II) are not effective in replacing RNase T and RNase PH.

Taken together, it is clear that strict control of intracellular levels of PAP I is critical for cell survival. In fact, *pcnB* expression in *E. coli* is regulated possibly in more than one way. At the translational initiation level, the presence of an AUU initiation codon and a poor ribosome binding site have been shown to limit the PAP I synthesis (17). Furthermore, phylogenetic evidence suggests that the PAP I homologs in many Proteobacteria are also possibly translated from AUU initiation codons (17). In addition, the *E. coli pcnB* coding sequence also contains at least four Shine-Dalgarno-like sequences that have been shown to be responsible for translational pausing, thereby limiting protein synthesis (42). These factors clearly reduce *pcnB* expression such that there are only ~30–50 molecules of PAP I present in exponentially growing *E. coli* cells (16).

The data reported here also raise the interesting question as to what selective advantage, if any, does the presence of PAP I provide in *E. coli*. Although it has been shown that polyadenylation of a mutant tRNA leads to its degradation (23), mature tRNAs are not likely substrates for polyadenylation in wild-type strains (19). Furthermore, it is not clear whether non-defective polyadenylated mature tRNA species suffer the same fate as polyadenylated mutant tRNA species. Moreover, the CCA adding enzyme tRNA nucleotidyltransferase has been shown to perform a dual role in tRNA biogenesis in both prokaryotes and eukaryotes. Besides repairing and adding the CCA trinucleotide to non-defective tRNAs, it can also prevent defective tRNAs from CCA maturation by adding CCACCA to defective tRNAs as part of a quality control mechanism (35,43). However, we have not observed any such additions to mature tRNAs ending with additional A residues [poly(A) tail] in *E. coli*, indicating that such tRNAs although defective for aminoacylation may well become functional after further, albeit slow, maturation (19).

One possibility is that limited polyadenylation of pre-tRNAs increases the probability that the bulk of the pre-tRNAs will be processed by RNase T, as polyadenylation of pre-tRNAs slows down their processing by RNase D, RNase BN/Z and possibly RNase PH (19). This could be important because unlike RNase T, which is inhibited by C nucleotides in its active site (40,41), RNase D, RNase BN/Z and RNase PH (44) can degrade through the CCA trinucleotide, generating defective tRNAs that cannot be repaired by tRNA nucleotidyltransferase. Thus under physiological conditions, polyadenylation could lead to larger pools of functional tRNAs.

The data in Table 1 also raise the question as to why some tRNAs are more susceptible to polyadenylation than others. For example, in the RNase T mutant, the levels of aminoacylated $tRNA^{His}$, $tRNA^{Cys}$ and $tRNA^{Metm}$ fell more than 10-fold. In contrast, aminoacylated $tRNA^{Pro}$ levels remained unchanged, whereas $tRNA^{Ala}$, $tRNA^{Arg}$ and $tRNA^{Phe}$ were only reduced 2.8-5-fold (Table 1). Because all tRNAs have the same four nucleotide single-stranded extension at their 3′ termini, some other aspect of tRNA three-dimensional structure must play a role in PAP I substrate recognition. Overall, seven of the eighty-six tRNA transcripts in *E. coli*, which includes $tRNA^{Pro}$, are not polyadenylated even when the synthesis of PAP I is deregulated (19).

Finally, it should be noted that the data presented here provide another clear distinction between polyadenylation in prokaryotes and eukaryotes. In eukaryotes, polyadenylation by canonical PAPs promotes mRNA stability and translation efficiency (9,10), whereas poly(A) tails added by non-canonical PAPs (*i.e.* Trf4, Trf5, PAPD5, etc.) destabilize transcripts as part of RNA surveillance mechanisms (45–47). In the absence of a canonical PAP in *E. coli*, polyadenylation of mRNAs by the non-canonical PAP I is believed to be a quality control mechanism targeting mainly RNA processing products,

breakdown products and defective RNA molecules (23). Although the increase in stabilities of many mRNAs in *E. coli* in the absence of PAP I (6,11,12,16) has provided credibility to the hypothesis, increased protein synthesis from such stabilized mRNAs has not been reported, with the exception of GlmS (48). Overall, the data presented here indicate why the levels of poly(A) polymerase are kept low in *E. coli*. Specifically, low levels of PAP I protect mature tRNAs from polyadenylation as well as help to maintain normal functional tRNA levels and protein synthesis. It will be interesting to determine if polyadenylation in other prokaryotes functions in a similar manner.

ACKNOWLEDGEMENTS

The authors thank Jessica R. Petree for her technical assistance and Grzegorz Wegrzyn, University of Gdansk, Poland, for PAP I antibodies.

FUNDING

Funding for open access charge: National Institute of General Medical Sciences [GM57220 and GM81554 to S.R.K.].

Conflict of interest statement. None declared.

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