Polyadenylation helps regulate functional tRNA levels in Escherichia coli

Bijoy K. Mohanty, Valerie F. Maples and Sidney R. Kushner*

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

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INTRODUCTION

In Escherichia coli, diverse processing pathways employing RNase E, RNase P, polynucleotide phosphorylase (PNPase) and RNase II are used to generate pre-tRNA species from primary transcripts (1–5). Subsequently, the pre-tRNAs undergo additional maturation at either both their 3′- and 5′-ends or at their 3′-ends only (3–10) to generate functional tRNAs. It has been shown that RNase T, RNase PH, RNase D, RNase BN are the four 3′ → 5′ exoribonucleases that primarily participate in 3′-end maturation of tRNAs and many other stable RNAs (6,7,9), although RNase BN has endonucleolytic activity in vivo and is also called RNase Z (11–13).

Interestingly, E. coli can use some incompletely processed stable RNAs (tRNAs) without significant effects on their growth phenotype (8,14–16), but tRNAs are an exception. All tRNAs, which account for the major fraction of stable RNAs in the cell (17), must be completely processed at their 3′-termini before they can be aminoacylated and used in protein synthesis (18). Although the 3′-ends of some tRNA precursors are probably matured by a preferred set of exonucleases (3,5,9), it is thought that the majority of the 3′-end tRNA maturation is carried out by RNase T and RNase PH with only minor contributions by RNase D and RNase BN (9).

Taken together these results present a comprehensive overview of the processing of primary tRNA transcripts into functional species. However, the observation of polyadenylated tRNAs in the absence of both RNase T and RNase PH (16,19) was rather unexpected, particularly since polyadenylation in E. coli by poly(A) polymerase I (PAP I) has been almost exclusively characterized in relation to mRNAs (20–23). Furthermore, sequencing analysis of hisR, cysT and leuX transcripts has shown that a significant fraction (~20–33%) of the transcripts have short poly(A) tails in a RNase PH single mutant (4,5). Interestingly, the fraction of normal pre-tRNAs (not defective based on nucleotide sequence) with poly(A) tails was considerably higher than previously observed for other transcripts (mRNAs and rRNA) in E. coli (24,25).

Although no poly(A) tails have been detected on mature tRNAs or 5S rRNA in wild-type E. coli, Li et al. (26) proposed a model in which the primary function of polyadenylation was to identify and present defective tRNA processing intermediates for recycling through degradation pathway(s) that are part of a general quality control process. The evidence for poly(A)-dependent degradation of a mutant tRNA<sup>1<sub>Tyr</sub></sup> (26) and various mRNAs (22,24,27) provided support for this hypothesis, but it did not explain the presence of polyadenylated pre-tRNA transcripts in the rph-1 mutant that were not defective (4,5).

Similarly, it has been suggested that the shorter poly(A) tails observed on many stable RNA precursors resulted from degradation of longer poly(A) tails by exoribonucleases such as polynucleotide phosphorylase (PNPase), RNase II or RNase R (19). However, inactivating either PNPase or RNase II did not change the length of poly(A) tails associated with leuX transcripts.

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

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Materials and methods

Bacterial strains and plasmids

The *E. coli* strains used in this study were all derived from MG1693 (rph-1 thyA715) (E. coli Genetic Stock Center, Yale University). This strain contains no RNase PH activity and has reduced expression of pyrE because of a single nucleotide frameshift in the rph gene (28). A rph<sup>+</sup> derivative was constructed by transducing a P1 lysate grown on *E. coli* C600 into MG1693 and selecting for faster growing isolates on minimal medium. Several independent transductants were sequenced to confirm the presence of the wild-type rph coding sequence. One such isolate was designated SK10153 (thyA715). SK9124 [rph-1 thyA715/pBMK11 (penB<sup>R</sup>)] and SK10148 (Arnt::kan rph-1 thyA715) have been previously described (2,24). A P1 lysate grown on SK10148 was used to transduce SK10153 (thyA715) and SK10019 (pnpΔ683 rph-1 thyA715) to construct SK10592 (Arnt::kan thyA715) and SK10609 (Arnt pnpΔ683 rph-1 thyA715), respectively. SK10575 (Arnb Arnt rph-1 thyA715) was made by transduction with P1 phage grown on CMA201 (Arnb::tet<sup>R</sup> rph-1 thyA715) (29) into SK10148 (Arnt::kan rph-1 thyA715).

The ΔpenB::aac(3)-IV (apramycin, Apr<sup>R</sup>) deletion/substitution allele in SK4465 was obtained using the method of Hamilton et al. (30). Briefly, the penB coding sequence starting from amino acid six after the UUG translation start codon until two amino acids upstream of the translation stop codon was replaced by the aac(3)-IV apramycin resistance cassette obtained from plasmid pSET152 (Genbank Accession No. 414670). SK10593 [Arnt ΔpenB::aac(3)-IV] and SK10020 [Arnt ApenB::aac(3)-IV rph-1 thyA715] were constructed by transducing SK10592 and SK10148, respectively, with P1 grown on SK4465. Plasmid pBMK58 (rnt<sup>R</sup>/Ap<sup>R</sup>) was constructed by cloning the rnt coding sequence containing its own promoter into pWSK29 (31) at the BamHI–PstI sites. A PCR fragment containing the rnt coding sequence was amplified using primer pairs RNT-BAMH (175 nt upstream of ATG start codon) and RNT-PST (74 nt downstream of TAA) using Phusion<sup>TM</sup> high fidelity DNA polymerase (NEB). SK10589 (Arnt rph-1/ pBMK58) was constructed by transforming pBMK58 into SK10148.

Northern analysis, dot blots and poly(A) sizing assay

The details of the poly(A) sizing assay, RNA isolation, Northern analysis and dot blots have been described previously (32). All strains were grown to Klett 50 (~10<sup>8</sup> cells/ml, No. 42 green filter) for isolation of steady-state RNA. For RNA half-life experiments rifampicin (500 μg/ml) and nalidixic acid (40 μg/ml) were added to cultures at Klett 50 prior to harvesting the cells at selected times. For PAP I induction, IPTG (350 μmol) was added to cultures at Klett 50 and cells were collected at the specified time points. All RNAs were quantified by measuring the OD<sub>260</sub> using a Nanodrop (2000C) apparatus (ThermoFisher) and were initially analyzed on an 1% agarose gels in TAE (Tris–acetic acid–EDTA) to check their integrity. RNA concentrations were normalized to a wild-type culture (32). For Northern analysis, total RNA (12 μg for tRNAs, 2.5 μg for SS rRNA and 5 μg for lpp mRNA per lane) was separated in 6% polyacrylamide gels containing 8 M urea in TBE (Tris–borate–EDTA buffer) and transferred to a positively charged nylon membrane (Nytran<sup>®</sup> SPC, Whatman<sup>®</sup>). Poly(A) sizing assays were carried out using 20% polyacrylamide gels.

Western blotting

Cells from 50 ml of exponentially growing cultures were harvested by centrifugation at 5000 rpm for 5 min at 4°C in a Beckman–Coulter<sup>TM</sup> (Avanti<sup>TM</sup> J-25) centrifuge. Cell pellets were resuspended in 300 μl of CellLytic<sup>TM</sup> B Bacterial Cell Lysis/Extraction Reagent (Sigma<sup>®</sup>) containing protease inhibitors (SIGMAFAST<sup>TM</sup> Protease Inhibitor Cocktail, EDTA free, Sigma<sup>®</sup> and 1 mM PMSF) and stored on ice. Cells were disrupted by sonication (Misonix, S-4000) and crude extracts were clarified by centrifugation in a microcentrifuge at full speed for 15 min at 4°C. Protein samples were quantified by Bradford assay using a Nanodrop (2000C) apparatus. Protein samples (125 μg) were separated on SDS-polyacrylamide (10%) gel and electrotransferred to PVDF membrane (Immobilon<sup>TM</sup>-P, Millipore) using a Bio-Rad Mini-Protean 3 gel apparatus. Antibodies against PAP I (kindly provided by Grzegorz Wegzryn, University of Gdansk, Poland) were initially incubated in 750 μg of cell lysate obtained from a penB deletion strain for 1 h and used to probe the membrane using Pierce<sup>®</sup> ECL Western Blotting substrate. The protein bands were detected and quantified using a G:Box (Syngene).
Determination of aminoacylation levels of tRNAs

All the strains were grown with shaking to Klett 50 at 37°C. Cells from 5 ml of culture were collected at 4°C and total RNA was isolated under acidic condition essentially as described by Varshney et al. (33). In one set of RNA samples (3 μg each), tRNA was decayed by treatment with 0.5 M Tris–HCl (pH 9.0) in a total volume of 40 μl for 30 min at 37°C. The RNA was precipitated at −20°C by adding 1/10th volume of 2 M sodium acetate (pH 5.2), 1 μl of GlycoBlue™ (Ambion) and 2.5 volume of 100% ethanol. The RNA was pelleted by centrifugation for 15 min at 4°C. The pellets were dissolved in the original volume of 10 mM sodium acetate (pH 4.5) and 1 mM Na2EDTA. Both untreated and Tris treated RNA samples (3 μg each) were mixed with an equal volume of loading buffer and separated on a 6.5% acid urea gel in the cold room as described by Varshney et al. (33) with following modifications. The electrophoresis was carried out at 250 V for ~8 h using a gel (19 cm long) until the xylene cyanol dye front was ~3–4 cm from the bottom of the gel. The RNA was transferred to a positively charged nylon membrane as described previously (32).

RNase H treatment

Total RNA (5–12 μg) was treated with RNase H (USB) in presence of oligo-d(T)20 to remove poly(A) tails as described previously (34).

Probe selection, hybridization of membranes and quantification of data

Oligonucleotide probes (Supplementary Table S1) were selected to hybridize to the mature sequences of all tRNAs and 5S rRNA. Various tRNA isotypes were differentiated based on differences in their mature sequences. Oligonucleotide probes were 5'-end labeled using T4 polynucleotide kinase (NEB) and γ-32P-ATP (Perkin Elmer) as described elsewhere (32). In most cases, the same membrane was probed multiple times after stripping the previous probe as previously described (32). The lpp mRNA was detected by probing with 32P-labeled lpp DNA fragments as described previously (23). All Northern blots and poly(A) sizing gels were scanned using a PhosphorImager (GE Healthcare, Storm™ 840). All data were quantified using ImageQuant TL 5.2 software (GE Healthcare).

RT-PCR cloning and sequencing of 5'-3' ligated transcripts

The 5'- and 3'-ends of tRNAs and 5S rRNA were identified by cloning and sequencing the reverse transcription-PCR products obtained from 5'- to 3'-end-ligated circular RNAs following the methods described previously (32). The 5’-3’ junctions of the cDNAs were amplified with a pair of gene-specific primers using GoTaq®Green Master Mix (Promega).

RESULTS

The level of short poly(A) tails (<10 nt) increases dramatically in an RNase T RNase PH double mutant

Since some of the tRNA precursors in an RNase T RNase PH double mutant as well as 5S and 23S rRNA precursors in RNase T single mutants have been shown to contain short poly(A) tails (16,19,35), we hypothesized that PAP I was significantly involved in stable RNA metabolism. Accordingly, we carried out dot blot experiments to determine the total poly(A) level in a RNase T RNase PH double mutant. We were surprised to find an ~50% reduction in total poly(A) level compared to a wild-type control (data not shown). However, since dot blots do not detect poly(A) tails <15 nt in length, we performed a poly(A) sizing assay, which can visualize poly(A) tails as short as a single nucleotide.

The polyadenylation profiles of a wild-type control and a RNase PH single mutant were almost identical, although a small but reproducible increase in total poly(A) level spanning all size ranges was observed in an rph-1 strain (Figure 1, lanes 2 and 3; Table 1). In contrast, in an RNase T single mutant there was ~2.5-fold decrease in the amount of longer poly(A) tails (>10 nt) accompanied by a 4-fold increase in the level of short poly(A) tails (<10 nt) (Figure 1, lane 4 and Table 1) compared to both the wild-type and rph-1 strains with the majority of the increase occurring in the range of 1–6 nt (Figure 1, lane 4). In the absence of both RNase T and RNase PH, there was a dramatic increase in the level of 4- to 9-nt long poly(A) tails compared to the Δmrt single mutant (Figure 1, lane 5). Overall, there was ~30-fold increase in the amount of short poly(A) tails (<10 nt) in the RNase T RNase PH double mutant compared to the wild-type control (Figure 1, lanes 2 and 5; Table 1). Interestingly, the level of longer poly(A) tails (>10 nt) remained almost unchanged in the RNase T RNase PH double mutant compared to the RNase T single mutant (Figure 1, lanes 4 and 5; Table 1). Overexpression of RNase T through the introduction of pBMK58 (a 6- to 8-copy number plasmid carrying the rnt under the control of its own promoter) into the RNase T RNase PH double mutant led to a polyadenylation profile that was identical to the wild-type control (data not shown).

The short poly(A) tails observed in RNase T mutants are synthesized by PAP I

PAP I (encoded by pcnB) is the major polyadenylating enzyme in E. coli (22,24,25), although PNPase also adds heteropolymeric tails to a limited extent (36,37). As shown in Figure 1 (lane 6), deletion of pcnB led to the disappearance of all the longer (>10 nt) poly(A) tails as well as the vast majority of the smaller ones (<10 nt), indicating that the tails were added primarily by PAP I in both the RNase T single (data not shown) and the RNase T RNase PH double mutant. The few remaining small tails (<6 nt) in the Δmrt ΔpcnB rph-1 triple mutant (Figure 1, lane 6) most likely arose from either the biosynthetic activity of PNPase or chromosomally encoded. As that were present at the 3’-ends of RNA breakdown products (36).
In order to determine if the 30-fold increase in the level of short poly(A) tails in the RNase T RNase PH double mutant was due to increased levels of PAP I protein, western-blot analysis was carried out. PAP I protein levels increased between 1.8- and 2.8-fold in all the mutants compared to the wild-type control (Figure 2). The increase in PAP I protein level in the rph-1 single mutant was consistent with the increase in the level of poly(A) tails across all size ranges compared to the wild-type control (Figure 1, lanes 2 and 3; Table 1). However, the increases in PAP I protein in the Drnt single and Drnt rph-1 double mutants were less than what was seen in the rph-1 single mutant, even though there were 4- and 30-fold increases in short poly(A) tails in the respective strains (Figure 1, lanes 4 and 5; Table 1).

Increased levels of short poly(A) tails result from polyadenylation of tRNA and 5S rRNA precursors

The tRNA concentration in exponentially growing E. coli has been estimated to be ~0.5 mM (2–3 × 10^5 molecules per cell), which constitutes up to 20% of the total RNA (38–40). Although previous observations have shown that some tRNAs and rRNAs precursors contained short poly(A) tails in the absence of either RNase PH or RNase T or both (4,5,8,9,16), these results were never quantitatively analyzed to determine if the polyadenylation profiles of specific species changed in various mutants as shown in Figure 1. We hypothesized that the extensive increase in poly(A) levels in Drnt rph-1 double mutant resulted from extensive polyadenylation of unprocessed 3'-ends of tRNAs and 5S rRNA, which were normally substrates for RNase T and PH (9,19).

Accordingly, we quantitatively analyzed a series of tRNAs, as well as 5S rRNA, in strains defective in various combinations of RNase T, RNase PH and PAP I using Northern blots. In an initial study of 14 tRNA species, the absence of either RNase PH or RNase T alone had no significant effect on maturation (Figure 3, lanes 1–3). In contrast, in the absence of both RNase T and RNase PH, there were significant amounts of higher molecular weight species presumed to be the immature precursors in the case of leuX, hisR, pheU, pheV, cysT, metT and metU (Figure 3, lane 4). Surprisingly, in the case of the three proline tRNAs (proK, proL and proM) and the metf1 and f2 isotypes (metV, metW, metY and metZ) inactivation of RNase T and RNase PH did not result in the accumulation of any detectable immature species (Figure 3, lane 4). In the case of 5S rRNA, the absence of RNase T alone led to a quantitative shift to a larger species (Figure 3, lane 3), in agreement with

### Table 1. Poly(A) tail distribution in various strains

| Strain          | Genotype                       | Relative amounts of poly(A) tails |
|-----------------|--------------------------------|----------------------------------|
| SK10153         | thyA715                        | <10 nt: 1                        |
| MG1693          | rph-1 thyA715                  | >10nt: 1                         |
| SK10592         | Drnt thyA715                   | <10 nt: 4 ± 1                    |
| SK10148         | Drnt rph-1 thyA715             | >10nt: 0.4 ± 0.1                 |
| SK10020         | ApcnB Drnt rph-1 thyA715       | <10 nt: 0.6 ± 0.2                 |
|                 |                                | >10nt: ND                         |

*The level of poly(A) tails was determined by separately integrating all bands >10 nt and <10 nt as shown in Figure 1 using ImageQuant TL 5.2 software (see ‘Materials and Methods’ section). The pixel counts for the respective group of bands in the wild-type control were set at one. ND = not detected.
previous work that has shown that RNase T was essential for removing the three extra nucleotides from the 3’-terminus of the p5S rRNA species (16).

The immature species observed with the leuX, hisR, pheU, pheV, cysT, metT and metU tRNAs and 5S rRNA in the Δrnt Δrph-1 double mutant were not discrete in size and appeared to vary in length by a small number of nucleotides (Figure 3, lane 4). Since previous work indicated that some immature tRNA and 5S rRNA precursors were polyadenylated (19), we also analyzed the pattern of precursors in a Δrnt Δrph-1 ΔpcnB triple mutant. In some cases, inactivation of PAP I activity led to an almost complete loss of the immature species (leuX and cysT, Figure 3, lane 5). With other tRNAs, (hisR, pheU, pheV, metT and metU) some immature species were still observed but they were shorter than those seen in the Δrnt Δrph-1 double mutant (Figure 3, lane 5). In the case of 5S rRNA, the polyadenylated species in the Δrnt Δrph-1 mutant (Figure 3, lane 4) disappeared completely, but the intermediate containing three extra nucleotide at the 3’-terminus remained (Figure 3, lane 5).

In order to provide further support for our hypothesis that the > 30-fold increase in poly(A) levels observed in the Δrnt Δrph-1 double mutant resulted primarily from polyadenylation of tRNA precursors, we expanded our study to include 50 of the 86 E. coli tRNA genes (Table 2). Overall 24 distinct tRNA isotypes as well as one rRNA encoded by 58 different genes were quantitatively examined (Table 2). Only seven tRNA transcripts out of 58 genes studied (12%) were not affected by the loss of both RNase T and RNase PH (Table 2). In contrast, the precursors for the vast majority of tRNAs (43 of 50) and 5S rRNA accumulated in the Δrnt Δrph-1 double mutant. The level of tRNA precursor accumulation varied from as little as 20% to as high as 71% compared to the wild-type control (Table 2). Furthermore, the levels of precursor tRNAs were significantly reduced (8–59%) in the ΔpcnB Δrnt Δrph-1 triple mutant compared to the Δrnt Δrph-1 double mutant supporting a direct role for PAP I.

Interestingly, the absence of PAP I had a variable effect on the reduction of the fraction of immature tRNA species (both polyadenylated and non-polyadenylated). Although the fraction of immature species for most of the tRNAs studied was reduced between 1.1- and 2.7-fold, for some prec-tRNA species (Arg2, Asn and Ser5) the fraction of immature species was reduced between 3.2- and 4-fold compared to the Δrnt Δrph-1 double mutant. In the case of 5S rRNA, the level of precursors was 100%, as expected based on previously published work (16). The seven tRNAs that did not use either RNase T or RNase PH for their 3’-end maturation were also not substrates for PAP I (Table 2).

Polyadenylated tRNA and 5S rRNA precursors have significantly altered half-lives

Since it has been argued that polyadenylation functions as part of the cell’s RNA quality control pathway (26), we reasoned that the polyadenylated species observed in Figure 3 should have significantly shorter half-lives than...
their comparable mature species. This idea would be consistent with previous reports showing that polyadenylated mRNAs and small regulatory RNAs (sRNAs) have shorter half-lives compared to the non-polyadenylated species (22, 24, 27, 41–45).

Accordingly, we measured the half-lives of 16 tRNAs and 5S rRNA (Table 3). The mature species for all the tRNAs tested had half-lives >120 min in the wild-type control. In fact, the level of most of the mature tRNAs actually increased steadily during wild-type control. In fact, the level of most of the tRNAs tested had half-lives shorter compared to the non-polyadenylated species.

Table 2. Effect of absence of RNase T and RNase PH on selected tRNAs and rRNA

| tRNA isotype/rRNA | No. of Loci | Gene(s) | Percentage of immature transcriptsa | Presence of poly(A) tailsb |
|------------------|-------------|---------|-----------------------------------|---------------------------|
| Arg2             | 4           | argQ, argY, argY, argZ            | 45 ± 3                | 14 ± 2                    |
| Asn              | 4           | asnT, asnU, asnV, asnW            | 27 ± 4                | 8 ± 2                     |
| Cys              | 1           | cysT                              | 44 ± 0                | 31 ± 2                    |
| Glu2             | 4           | gltT, gltU, gltV, gltW            | 26 ± 3                | 17 ± 0                    |
| His              | 1           | hisR                              | 71 ± 2                | 59 ± 1                    |
| Leu1             | 4           | leuT, leuQ, leuP, leuV            | 41 ± 1                | 20 ± 3                    |
| Leu2             | 1           | leuU                              | 20 ± 2                | 12 ± 2                    |
| Leu3             | 1           | leuW                              | 54                   | 44                        |
| Leu4             | 1           | leuZ                              | 38                   | 29                        |
| Leu5             | 1           | leuX                              | 61 ± 1                | 30 ± 5                    |
| Lys              | 6           | lysQ, lysT, lysV, lysW, lysY, lysZ | 24                   | 9                         |
| Met f1, Met f2   | 4           | metZ, metW, metV, metY            | 0                    | 0                         |
| Met m            | 2           | metT, metU                        | 58 ± 0                | 41 ± 3                    |
| Phe              | 2           | pheU, pheV                        | 67 ± 3                | 45 ± 1                    |
| Pro1, Pro2, Pro3 | 3           | proK, proL, prom                  | 50                   | 23                        |
| Ser1             | 1           | serT                              | 56                   | 42                        |
| Ser2             | 2           | serW, serX                        | 36                   | 9                         |
| Val1             | 5           | valU, valX, valY, valT, valZ      | 53                   | 48                        |
| Val2A, Val2B     | 2           | valY, valW                        | 33                   | 38                        |
| 5S               | 8           | rrfA, B.C,D,E,F,G,H               | 100 ± 0               | 100 ± 0                   |

aRepresents the fraction of immature species out of total (mature, immature, and polyadenylated species, see Figure 3) RNA species detected. Data, with standard deviations, represent the average of at least three independent experiments. bPoly(A) tails were scored as being present if the immature and/or polyadenylated precursor species (IM), beyond 0-min time point (70 s after rifampicin addition).

Table 3. Half-lives of tRNAs and 5S rRNA in various genetic backgrounds

| Transcripts          | Wild-type | Arnt rph-1 | ApenB Arnt rph-1 |
|----------------------|-----------|------------|-----------------|
| cysT                 | IM        | 90 ± 10    | NP              |
|                      | M         | ND (†)     | ND (†)          |
| hisR                 | IM        | 80 ± 5     | 100 ± 5         |
|                      | M         | ND (†)     | ND (†)          |
| leuT, leuQ, leuP, leuV| IM        | 77 ± 10    | NP              |
|                      | M         | ND (†)     | ND (†)          |
| leuW                 | IM        | 153        | NC              |
|                      | M         | ND (†)     | ND (†)          |
| gltT, gltU, gltV, gltW| IM        | 133 ± 10   | NP              |
|                      | M         | ND (†)     | ND (†)          |
| proK, proL, proM     | IM        | 70         | NC              |
|                      | M         | ND (†)     | ND (†)          |

The data for the 5S rRNAa indicates the band intensities increased up to 120 min.

Supplementary Table S1). Similar increases in mature tRNA levels after rifampicin addition have been previous-
mutant steadily increased during the experiments, similar to what was observed for the wild-type control (Figure 4, lanes 11–15; Table 4). The level of mature 5S rRNA species increased slightly in the wild-type strain during half-life experiment (Figure 4, lanes 1–5; Table 3 and Supplementary Figure S1). In contrast, the levels of both the immature species in the Drnt rph-1 mutant decreased indicating their instability (Figure 4, lanes 6–10, Supplementary Figure S1). The polyadenylated species IM1 decreased at a faster rate with a half-life of 100 min compared to the non-polyadenylated species IM2 with a half-life of 120 min (Table 4).

However, the level of the immature species in Drnt Drnt DpcnB rph-1 mutant increased under similar experimental conditions (Figure 4, lanes 11–15, Supplementary Figure S1). The absence of polyadenylation improves aminoacylation levels and growth rate in an Drnt DpcnB rph-1 triple mutant

The extent of aminoacylation of mature tRNAs is an important factor in maintaining the normal growth rate in any organism. Considering that a significant fraction of most tRNAs were immature and polyadenylated in the Drnt rph-1 double mutant (Table 2), we assumed that these species were not charged, resulting in poor growth. In contrast, since the inactivation of PAP I led to decreases in immature tRNA species along with concomitant increases in mature tRNAs (Table 2 and Figure 4), we predicted an increased level of aminoacylation and a shorter generation time in the triple mutant compared to

Table 4. Percentage of tRNA aminoacylation in various strains

| tRNA isotypes/genes | Wild-type | rph-1 | Drnt | Drnt rph-1 | DpcnB Drnt rph-1 |
|---------------------|------------|-------|------|------------|-----------------|
| Arg2/argQ, argV, argY, argZ | 71 ± 5 | 73 ± 2 | 71 ± 5 | 33 ± 1 | 58 ± 4 |
| His/hisR | 76 ± 1 | 70 ± 5 | 70 ± 5 | 18 ± 4 | 36 ± 4 |
| Leu5/leuX | 91 ± 1 | 89 ± 1 | 88 ± 1 | 38 ± 2 | 79 ± 3 |
| Metm/metT, metU | 85 ± 1 | 82 ± 2 | 80 ± 2 | 33 ± 3 | 56 ± 5 |
| Cys/cysT | 60 ± 3 | 52 ± 3 | 54 ± 3 | 26 ± 2 | 37 ± 2 |
| Lys/lysQ, lysT, lysV, lysW, lysY, lysZ | 70 ± 3 | 71 ± 3 | 69 ± 3 | 30 ± 2 | 54 ± 3 |
| Pro/proK, proL, proM | 75 ± 1 | 78 ± 1 | 74 ± 1 | 74 ± 2 | 75 ± 2 |

The percentage of aminoacylated tRNAs were determined as described in Figure 5. The numbers presented are the average of three independent determinations.

The absence of polyadenylation improves aminoacylation levels and growth rate in an Drnt DpcnB rph-1 triple mutant

Figure 4. Northern blots to determine the half-lives of various tRNA and 5S rRNA transcripts. Total RNA from SK10153 (wild-type), SK10148 (Drnt rph-1) and SK10020 (Drnt DpcnB rph-1) was isolated at times (minutes after rifampicin addition) indicated at the top of the blot and was separated on 6% PAGE, transferred to a positively charged nylon membrane and probed with transcripts specific probes as described in the ‘Materials and Methods’ section. The blot was scanned using a STORM 840 PhosphoImager (GE Healthcare) and the intensities of the bands were quantified using ImageQuant TL software. The values (log of the percentage of transcript remaining) were plotted as a function of time (Supplementary Figure S1) to calculate each half-life (Table 3). M, IM, IM1 and IM2 are as described in the legend to Table 3.
the Arnt rph-1 double mutant. To test this hypothesis, we measured in vivo aminoacylation of selected tRNAs using the method of Varshney et al. (33). This method allows the isolation of tRNAs from exponentially growing cells and the separation of charged and uncharged tRNA species on polyacrylamide acid urea gels (Figure 5).

The levels of charged tRNAs in the wild-type strain were found to be in the range between 60% and 91% (Table 4 and Figure 5). These results were consistent with previous reports on aminoacylation levels of several tRNAs such as cysT (33, 46–49). As predicted, all aminoacylation levels of all tRNAs studied are reported in Table 4.

Figure 5. Representative Northern blots showing the determination of the percentage of aminoacylated tRNAArg2 and tRNAPro. Total RNA isolated from SK10153 (wild-type), MG1693 (rph-1), SK10592 (Arnt), SK10148 (Arnt rph-1) and SK10020 (Arnt ApcnB rph-1) were untreated (lanes 1–5) or treated with 0.5 M Tris (pH 9) to chemically deacetylate tRNAs (lanes 6–10) and were 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 immature tRNAs [with or without poly(A) tails]. The net extent of aminoacylation (lanes 1–5) for band II was calculated as percentage of total counts (bands I + II) minus the percentage of high molecular weight species of total counts (bands I + II) after Tris treatment in lanes 6–10 (33). The aminoacylation levels of all tRNAs studied are reported in Table 4.

With regard to generation times, inactivation of RNase T and RNase PH had no significant effect (Table 5), while the loss of RNase T activity led to small but significant increase in generation time (Table 5). In contrast, in the absence of both RNase T and RNase PH the generation time of the double mutant increased over 2-fold (25 versus 56 min, Table 5). The generation times of the ApcnB and ApcnB rph-1 mutants were identical and were only marginally slower compared to the wild-type or rph-1 single mutant strains (Table 5). However, although the Arnt single mutant and the Arnt ApcnB double mutant had somewhat similar growth rates, the generation time of the ApcnB Arnt rph-1 triple mutant improved significantly compared to the Arnt rph-1 double mutant (Table 5).

Table 5. Generation times of various strains in Luria broth

| Strains                  | Genotype       | Generation time (min) |
|-------------------------|----------------|-----------------------|
| SK10153                 | thyA715 (wild-type) | 25 ± 1                |
| MG1693                  | rph-1 thyA715  | 27 ± 1                |
| SK10592                 | Arnt thyA715  | 38 ± 2                |
| SK10148                 | Arnt rph-1 thyA715 | 56 ± 2                |
| SK10591                 | ApcnB thyA715 | 30 ± 1                |
| SK4465                  | ApcnB rph-1 thyA715 | 30 ± 1                |
| SK10593                 | ApcnB Arnt thyA715 | 35 ± 2                |
| SK10020                 | ApcnB Arnt rph-1 thyA715 | 48 ± 2                |

*Generation times were measured at 37°C and represent the average of at least two independent experiments.

With regard to generation times, inactivation of RNase T and RNase PH had no significant effect (Table 5), while the loss of RNase T activity led to small but significant increase in generation time (Table 5). In contrast, in the absence of both RNase T and RNase PH the generation time of the double mutant increased over 2-fold (25 versus 56 min, Table 5). The generation times of the ApcnB and ApcnB rph-1 mutants were identical and were only marginally slower compared to the wild-type or rph-1 single mutant strains (Table 5). However, although the Arnt single mutant and the Arnt ApcnB double mutant had somewhat similar growth rates, the generation time of the ApcnB Arnt rph-1 triple mutant improved significantly compared to the Arnt rph-1 double mutant (Table 5).

Table 5. Generation times of various strains in Luria broth

| Strains                  | Genotype       | Generation time (min) |
|-------------------------|----------------|-----------------------|
| SK10153                 | thyA715 (wild-type) | 25 ± 1                |
| MG1693                  | rph-1 thyA715  | 27 ± 1                |
| SK10592                 | Arnt thyA715  | 38 ± 2                |
| SK10148                 | Arnt rph-1 thyA715 | 56 ± 2                |
| SK10591                 | ApcnB thyA715 | 30 ± 1                |
| SK4465                  | ApcnB rph-1 thyA715 | 30 ± 1                |
| SK10593                 | ApcnB Arnt thyA715 | 35 ± 2                |
| SK10020                 | ApcnB Arnt rph-1 thyA715 | 48 ± 2                |

*Generation times were measured at 37°C and represent the average of at least two independent experiments.

Figure 5. Representative Northern blots showing the determination of the percentage of aminoacylated tRNAArg2 and tRNAPro. Total RNA isolated from SK10153 (wild-type), MG1693 (rph-1), SK10592 (Arnt), SK10148 (Arnt rph-1) and SK10020 (Arnt ApcnB rph-1) were untreated (lanes 1–5) or treated with 0.5 M Tris (pH 9) to chemically deacetylate tRNAs (lanes 6–10) and were 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 immature tRNAs [with or without poly(A) tails]. The net extent of aminoacylation (lanes 1–5) for band II was calculated as percentage of total counts (bands I + II) minus the percentage of high molecular weight species of total counts (bands I + II) after Tris treatment in lanes 6–10 (33). The aminoacylation levels of all tRNAs studied are reported in Table 4.

With regard to generation times, inactivation of RNase T and RNase PH had no significant effect (Table 5), while the loss of RNase T activity led to small but significant increase in generation time (Table 5). In contrast, in the absence of both RNase T and RNase PH the generation time of the double mutant increased over 2-fold (25 versus 56 min, Table 5). The generation times of the ApcnB and ApcnB rph-1 mutants were identical and were only marginally slower compared to the wild-type or rph-1 single mutant strains (Table 5). However, although the Arnt single mutant and the Arnt ApcnB double mutant had somewhat similar growth rates, the generation time of the ApcnB Arnt rph-1 triple mutant improved significantly compared to the Arnt rph-1 double mutant (Table 5).

Overexpression of either RNase PH or RNase T from multicopy plasmids [pPP1 (rph+ Ap+) and pBMK58 (rnt+ Ap+)], respectively, restored the generation time of the Arnt rph-1 double mutant to the wild-type levels (data not shown).

RNase T prevents the polyadenylation of mature tRNA transcripts

Having implicated RNase T and RNase PH in the regulation of tRNA and 5S rRNA polyadenylation levels, we next determined the polyadenylation profile and exact location of poly(A) tail additions for selected single copy tRNAs such as hisR (Figure 6), cysT (Figure 6) and leuX (data not shown). In the case of the hisR and cysT pre-tRNAs, there are one to two additional nucleotides downstream of their mature 3'-termini (Figure 6), which arise from RNase E endonucleolytic cleavages of polycistronic transcripts (1, 2, 4). In contrast, the monocistronic leuX transcript is processed at the 3'-terminus primarily by PNPase leaving between 1- and 3-nt downstream of the CCA terminus (5).

We have previously shown that ~66% of the hisR, cysT and leuX transcripts cloned and sequenced from an rph-1 strain using 5'- to 3'-end ligated transcripts had immature 3'-ends (4, 5). About 20–33% of these transcripts also contained poly(A) tails up to 5 nt in length, but none of them were located at the mature 3'-terminus. Using this same method, the number of hisR, cysT and leuX transcripts with immature 3'-ends in the wild-type control (SK10153) was only ~30%, but between 8% and 23% had poly(A) tails (Figure 6; data not shown). Surprisingly, ~20% (6/30) of the hisR, ~12% (3/26) of cysT (Figure 6) and ~17% (3/18) of leuX (data not shown) transcripts were found to have 3' truncated sequences (5–16 nt upstream of the CCA terminus) without having poly(A) tails. In the Arnt rph-1 double mutant, all of the sequenced hisR, cysT and leuX transcripts (119 total) had immature 3'-ends (Figure 6; data not shown). Furthermore, the percentage of transcripts with poly(A) tails of up to 5 nt in
length increased to 80% (48/61) for hisR and 53% (17/32) for cysT (Figure 6). For the majority of the transcripts, the poly(A) tails were at least 1–2 nt downstream of the mature CCA termini (Figure 6). The origin of the single adenine 2-nt downstream of the CCA determinant (a1 in Figure 6) in the hisR clones could not be unequivocally determined because of the presence of a chromosomally encoded A at that location (Figure 6). Interestingly, some of the sequenced transcripts (5/61 for hisR and 4/32 for cysT) had poly(A) tails added immediately following the CCA determinant, which had not been previously seen in either wild-type (Figure 6) or rph-1 strains (3,4). Again, none of the poly(A) tails added to the CCA determinant were >5 nt. It should also be noted that although >50% of the clones were mature tRNAs in the wild-type control, no mature tRNAs were isolated from the rnt rph-1 double mutant (Figure 6).

We also sequenced 5S rRNA species derived from wild-type, Arnt and Arnt rph-1 strains (Figure 7). This stable RNA is generated endonucleolytically by RNase E cleavages of a larger 9S rRNA precursor (50), creating a p5S RNA species that contains an extra 3 nt at each end (51) (Figure 7). Although the 5' maturation process is still unknown, the 3 nt at the 3'-end are removed by RNase T (16). Fifty percent (10/20 clones) of the transcripts in the wild-type strain had both 5' and 3' mature ends (Figure 7). The rest of the clones had an additional 1–3 nt at both ends. As expected, all the transcripts derived from the Arnt single mutant (22/22 clones) had immature 3'-ends, although 41% (9/22 clones) had mature 5'-ends (Figure 7). Additionally, all of the transcripts had poly(A) tails that were 1–4 nt in length. While the first three As following C (A) may have been encoded, at least 9% (2/22 clones, small a) of the transcripts had poly(A) tails that were post-transcriptionally added. The number of post-transcriptionally added tails increased to 56% (15/27 clones, small a) and the length of the poly(A) tails increased from 4 to 7 nt in the Arnt rph-1 double mutant (Figure 7). It should also be noted that none of the pre-tRNAs (with or without poly(A) tails) and pre-5S rRNAs were defective based on their primary nucleotide sequences.

**Poly(A) tails at the 3'-ends of tRNAs are inherently short**

Previously we have identified poly(A) tails >17 nt associated with various mRNAs using an oligo(dT)$_{17}$ primed RT-PCR method (24,25,36,37). Unfortunately, this method does not allow one to determine the actual length of poly(A) tails and, more importantly, it discriminates against detection of poly(A) tails <15 nt. In
contrast, RT-PCR cloning using 5′- to 3′-end ligated transcripts is theoretically capable of detecting poly(A) tails of any length, but we were concerned that transcripts with poly(A) tails >5 nt might not ligate efficiently, resulting in the detection of only smaller poly(A) tails (Figures 6 and 7). Furthermore, it was also possible the short poly(A) tails were the result of very low in vivo PAP I levels (37).

Accordingly, in order to determine if the poly(A) tails added to tRNAs were inherently short, we used an alternative approach in which poly(A) tails >10–15 nt were removed by treating total RNA with RNase H in the presence of oligo(dT)20 (34). This method has been reliably employed by many laboratories to estimate the length of poly(A) tails in both prokaryotes and eukaryotes (34,52,53). In order to overcome the limitation of inherently low PAP I levels (37), we induced PAP I expression from a controlled expression plasmid [pBMK11/CmR, (24)] with IPTG for up to 45 min, circumstances under which we had previously detected long poly(A) tails on the lpp mRNA (24). As shown in Figure 8A, the total poly(A) level increased dramatically during the course of PAP I induction (lanes 3 and 5). Furthermore, pretreatment of the RNA with RNase H in presence of oligo(dT)20 led to an almost complete loss in detectable poly(A) tails (Figure 8A, lanes 4 and 6).

Subsequently, the blot was reprobed with hisR and cysT specific probes. After 15 min of PAP I induction, ~25–35% of both the transcripts showed an increase in size (~5–10 nt) (Figure 8A, lane 3 and data not shown) indicating the addition of short poly(A) tails. Furthermore, the fraction of larger tRNA transcripts increased >50% at the longer time of induction (Figure 8A, lane 5, data not shown). However, the larger transcripts were unaffected by the pretreatment with RNase H (hisR, Figure 8A, lanes 4 and 6; data not shown) indicating that the poly(A) tails on these transcripts were too short to hybridize to oligo(dT)20. As a control we examined the poly(A) tails associated with the lpp mRNA, since it has been shown that they increase in length after 15 min of PAP I induction (24). In fact, we observed a diffuse band of hisR transcripts following 15 min of PAP I induction (Figure 8B, lane 3). As expected, the band became more discrete after RNase H treatment such that it was similar to the bands from the uninduced strains (Figure 8B, lanes 1, 2 and 4).

**PNPase and RNase II do not regulate tRNA poly(A) tail length**

Li et al. (19) suggested that the short poly(A) tails they observed on tRNAs in the absence of RNase T or RNase PH arose from the shortening of longer tails by 3′ → 5′ exonucleases such as PNPase or RNase II. To test this hypothesis, we carried out a poly(A) sizing assay on Arnt rph-1, Arnt Arnb rph-1 and Arnt Δnpb rph-1 strains where we expected a shift to longer poly(A) tails compared to what was observed in the Arnt rph-1 double mutant (Figure 1, lane 5). In fact, there was a significant increase in the level of both short and long poly(A) tails in Arnt Arnb rph-1 and Arnt Δnpb rph-1 strains (data not shown), compared to the presence of primarily short poly(A) tails in the Arnt rph-1 strain (Figure 1, lane 5). The increases in the level of all poly(A) tails in both triple mutants was consistent with the role played by PNPase and RNase II in regulating the in vivo poly(A) tail levels (54).

Nevertheless, in order to determine if the increased levels of poly(A) tails in the two triple mutants were specific to tRNAs, Northern analysis was carried out on the hisR and leuX tRNAs, the two tRNAs with the highest level of immature species in the Arnt rph-1 double mutant (Table 2). Total RNA was pretreated with RNase H in the presence of oligo(dT)20 to evaluate the length of the poly(A) tails. In the absence of PNPase, both hisR and leuX had identical percentages (data not shown) as well as similar lengths of immature species compared to the Arnt rph-1 strain (Figure 9, lanes 1 and 5). However, in the absence of RNase II the hisR immature species were heterogeneous and the leuX immature species were slightly longer than in the Arnt rph-1 strain (Figure 9, lanes 1 and 3). The longer leuX immature transcript in the absence of...
polyadenylated species. FL = full-length.

...are noted above each lane. A star indicates immature and RNase H in the presence of oligo(dT20). Lanes 1 and 2, SK10148; lanes 3 and 4; SK10357; lanes 5 and 6, SK10609. Genotypes of each strain are noted above each lane. A star indicates immature and polyadenylated species. FL = full-length.

RNase II was consistent with its demonstrated role in the processing of the leuX downstream 3' sequences (5). More importantly, treatment with RNase H did not change the length of immature species compared to the non-treated samples in any genetic background (Figure 9, lanes 2, 4 and 6). An identical Northern analysis of 5S rRNA showed a minor effect of both RNase II and PNPase (Figure 9, lanes 4 and 6) in shortening the poly(A) tails. However, the overall poly(A) tail lengths associated with 5S rRNA remained short.

**DISCUSSION**

We have demonstrated here for the first time a role for polyadenylation in regulating the intracellular levels of functional tRNAs in *E. coli*. Essentially, RNase T and RNase PH, the two primary tRNA 3' processing ribonucleases compete with PAP I for the 3'-ends of pre-tRNAs (Figure 10). Since the PAP I level *in vivo* is comparatively low in wild-type cells [30–50 molecules/cell (37)], RNase T and/or RNase PH rapidly process the majority of pre-tRNAs in wild-type cells to generate mature tRNAs (Figure 10), which are rapidly aminoacylated making them no longer substrates for PAP I. Increased polyadenylation of tRNA substrates in the presence of excess PAP I (Figure 8) is consistent with this model.

In contrast, in the absence of both RNase T and RNase PH majority of the pre-tRNAs become substrates for polyadenylation by PAP I, generating significant levels of non-functional tRNAs (Figures 5 and 10; Table 4). Surprisingly, most of these species are processed very slowly (half-lives between 70 and 130 min, Table 3) presumably by the remaining tRNA processing enzymes such as RNase D, RNase BN/Z and RNase II (Figures 4 and 10). Only a small fraction of the polyadenylated species appear to be degraded via a general quality control process [Figures 4 and 10, (26)]. Of particular importance is the fact that the polyadenylation of pre-tRNAs occurs in wild-type cells (Figure 6), demonstrating that the competition among RNase T, RNase PH and PAP I is part of the normal tRNA processing pathway in *E. coli*.

Thus, the significant decreases in charged tRNA levels (Table 4) and increased cell generation time (Table 5) in the Δrnt rph-1 double mutant directly correlates with the 30-fold increase in poly(A) level (Figure 1 and Table 1). Furthermore, these defects are partially suppressed by inactivating PAP I (Tables 2–4). Although the activities of either RNase T or RNase PH appear to protect the vast majority of pre-tRNAs and mature tRNAs from PAP I mediated polyadenylation (Figure 3), the absence of RNase T alone leads to higher accumulation of short poly(A) tails (Figures 1 and 7) and significantly reduced growth rate (Table 2) compared to the absence of RNase PH. Taken together the above data indicated that RNase T was most effective in protecting the mature, stable RNA 3'-ends from polyadenylation.

Our data also show for the first time that poly(A) tail length *in vivo* is substrate dependent, indicating differential activity of PAP I on tRNAs and 5S rRNA versus other RNAs (mRNAs and 23S rRNA). For example, the poly(A) tails associated with tRNAs and 5S rRNA are predominantly short (<5 nt) (Figures 1 and 6–9) compared to what has been observed for mRNAs (Figure 8B) (22,24,25,36,37), although poly(A) tails with an average length of 7–9 nt have been reported for the rpsO mRNA (55). These results raise the question as to why the poly(A) tails added onto tRNAs, 5S rRNA (Figures 6 and 7) and other stable RNAs (19) are inherently short compared to those found on mRNAs.

One obvious explanation is that the short poly(A) tails on stable RNAs arise from rapid shortening of longer tails by PNPase or RNase II as previously proposed (19). This model is quite attractive because several studies have shown that these major 3'-5' exonucleases control the extent of polyadenylation of mRNAs (54,56,57). However, as shown in Figure 9, neither RNase II nor PNPase affected the length of the poly(A) tails associated with tRNAs and 5S rRNA. The data, however, are consistent with the requirement for ≥10 nt single-stranded regions for RNase II and PNPase to bind and degrade RNA substrates (56,58,59) as opposed to RNase T and RNase PH, which are very effective against single-stranded substrates up to 5–6 nt in length (60,61).

Moreover, it has been shown that Hfq, a RNA binding protein, affects PAP I processivity, both *in vitro* and *in vivo* (37,55,62). Interestingly, Hfq binds tRNAs with high specificity on its proximal surface, which is distinct.
transcription terminator following proM polyadenylation by PAP I and RNase II activity (10). In wild-type cells, the resulting generated by downstream RNase E cleavages are often trimmed back to 2–3 nt downstream of the CCA determinant by RNase II alone or by argX the polyadenylated fraction of polyadenylation reaction exists in E. coli (67), it is possible that a similar mech-
and DEAD box RNA helicases in (66). Since protein–protein interactions between PAP I
regulates the polyadenylation by the TRAMP complex (64). This multiprotein complex contains a
similar to what is observed with the TRAMP complex in S. cerevisiae (64). This multiprotein complex contains a
non-canonical PAP (Trf4p or Trf5p in S. cerevisiae, which is similar to E. coli PAP I), a Zn-knuckle protein
(Air2P or Air1P) and an RNA helicase (Mtr4p/Dob1p) (64). Furthermore, the complex has been shown to
target RNA substrates meant for further processing or exonucleolytic degradation by adding very short poly(A)
tails (~4–5 nt) (65). Of even more interest is the fact that recent data suggest that the RNA helicase component regulates the polyadenylation by the TRAMP complex (66). Since protein–protein interactions between PAP I and DEAD box RNA helicases in E. coli have already been demonstrated (67), it is possible that a similar mecha-
ism for controlling the extent of the tRNA polyadenylation reaction exists in E. coli, but is not employed with mRNAs (37).

Although it has been shown that the addition of short poly(A) tails to defective pre-tRNAs can initiate their degr-
adation (26) (Figure 10), the results presented in Figure 4 indicate that only a small fraction of the polyadenylated
 tRNA precursors are actually degraded. Rather, in the case of the hisR, cysT and leuX tRNAs, a significant portion of the polyadenylated tRNA precursors were actually converted into mature species with half-lives that ranged from 77 to 133 min (Table 3). This extremely slow conversion presumably is the result of very inefficient processing by some combination of RNase D, RNase BN/Z and RNase II (double question marks). Only a small fraction of the polyadenylated non-functional tRNAs are degraded, possibly by PNPase-dependent pathway (26).

from the poly(A) binding site (63). However, preliminary analysis comparing poly(A) tail profiles between Δarn
rph-1 and Δarn rph-1 hfq-1 mutants did not reveal any significant difference in tRNA tail lengths (data not shown). It is possible that the short poly(A) tails found on tRNAs and 5S rRNA are simply due to a more dis-
tributive mode of PAP I activity on tRNAs compared to a more processive mode that is used for mRNA
polyadenylation where Hfq plays a significant role.

Alternatively, the failure to elongate tRNA poly(A) tails, even after PAP I overexpression (Figure 8), could indicate a distinct polyadenylation reaction that may be similar to what is observed with the TRAMP complex in S. cerevisiae (64). This multiprotein complex contains a
non-canonical PAP (Trf4p or Trf5p in S. cerevisiae, which is similar to E. coli PAP I), a Zn-knuckle protein
(Air2P or Air1P) and an RNA helicase (Mtr4p/Dob1p) (64). Furthermore, the complex has been shown to
target RNA substrates meant for further processing or exonucleolytic degradation by adding very short poly(A)
tails (~4–5 nt) (65). Of even more interest is the fact that recent data suggest that the RNA helicase component regulates the polyadenylation by the TRAMP complex (66). Since protein–protein interactions between PAP I and DEAD box RNA helicases in E. coli have already been demonstrated (67), it is possible that a similar mecha-

Figure 10. A schematic model for processing of the E. coli argX operon. RNase E cleaves the polycistronic transcript to generate the individual pre-tRNAs (1,2) followed by RNase P cleavages, which generate the mature 3' ends on the pre-tRNAs. The mechanism for removal of the transcription terminator following proM is unknown (double question marks). The long 3' trailer sequences associated with some pre-tRNAs generated by downstream RNase E cleavages are often trimmed back to 2-3 nt downstream of the CCA determinant by RNase II alone or by polyadenylation by PAP I and RNase II activity (10). In wild-type cells, the resulting argX, leuT and hisR pre-tRNAs are quickly processed at their 3' ends by RNase T and RNase PH. PAP I adds poly(A) tail to any unprocessed pre-tRNAs, which are very low in abundance in wild-type cells [19], Figure 6). The mechanism of 3'-end maturation of proM is not clear (double question marks). In the absence of either RNase T or RNase PH, the polyadenylated fraction of argX, leuT and hisR pre-tRNAs increases significantly (4,5). However, in the absence of both the ribonucleases majority of the argX, leuT and hisR pre-tRNAs are polyadenylated (Figure 6) resulting in a large increase of non-functional tRNAs (Table 4). Most of these tRNAs are very slowly processed (Table 3) to chargeable mature species probably by the action of RNase D, RNase BN/Z and RNase II (double question marks). Only a small fraction of the polyadenylated non-functional tRNAs are degraded, possibly by PNPase-dependent pathway (26).
tails to pre-tRNAs is designed to slow down 3′-end processing by the enzymes that could destroy tRNA functionality, improving the chances of maturation by RNase T. The unique substrate specificity of RNase T (60), the most effective among all the tRNA processing enzymes, is consistent with this hypothesis.

Surprisingly, a small number of tRNAs (7/50) were found not to be the substrates for either RNase T or RNase PH (Table 2). These tRNAs were also not substrates for PAP I even in a Δmt rph-1 double mutant. Thus, no precursors of the proK, proL and proM tRNAs were observed in the absence of both RNase T and RNase PH (Figure 3, lanes 4 and 5). Even when PAP I was overexpressed these three tRNAs were not significantly polyadenylated (Mohanty and Kushner, manuscript in preparation). Although one can explain the lack of an RNase T and/or RNase PH effect based on the possibility that endonucleolytic cleavages occur immediately downstream of their CCA determinants, at this point it is not clear which endonuclease(s) might be carrying out this processing. Conversely, it is possible that these particular tRNAs are processed efficiently by exonucleases such as RNase D, RNase BN or RNase II.

On the other hand, it is more difficult to understand why only these seven tRNAs are not polyadenylated. All tRNAs have paired 5′- and 3′-ends, generally with a 4-nt single-stranded extension containing the CCA determinant, except for tRNA\textsuperscript{Metf1} and tRNA\textsuperscript{Metf2} (metY, metZ, metW and metV). All seven tRNAs resistant to polyadenylation have the same last 4-nt single-stranded extension (ACCA), but many of the tRNA species that are polyadenylated have the same 4-nt extension. However, there is one significant difference between the seven tRNAs that are not substrates for PAP I and the other 79 tRNA transcripts, namely a C nucleotide at the mature 5′-terminus. The vast majority of the tRNAs have G at their 5′-end with some having either an A (four species) or U (eight species) at this position. The presence of a 5′ C residue correlates perfectly with the 7/86 tRNAs that are resistant to polyadenylation, since the four \textit{asn} tRNAs have an U in the 5′ position and they are subject to polyadenylation and one of the species with an A as the 5′-terminus (\textit{trpT}) also gets polyadenylated (data not shown).

Finally, it has been demonstrated that overexpression of PAP I is toxic in a wild-type cell (24,70). Although the exact reason for this toxicity is still not well understood, the data presented in Figure 8, suggest that tRNAs are better PAP I substrates compared to mRNAs, considering the fact that after 15 min of PAP I induction >25% of the mature tRNA\textsuperscript{His} and tRNA\textsuperscript{Cys} were polyadenylated (Figure 8, data not shown). This is in contrast to what has been observed with mRNAs under similar circumstances in which the \textit{lpp} mRNA had the highest polyadenylation level of ~5% (25). The concomitant increase in the level of mature tRNA polyadenylation (Figure 8) and decreased cell viability with increased time of PAP I induction (24) suggest a direct link between reduced levels of functional tRNAs, slower growth and eventual cell death (24). Taken together, it appears that polyadenylation in \textit{E. coli} plays an important role in ensuring appropriate levels of functional tRNAs.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online: Supplementary Table 1 and Supplementary Figure 1.

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