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Endonucleolytic cleavages by RNase E generate the mature 3′ termini of the three proline tRNAs in *Escherichia coli*

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ABSTRACT

We demonstrate here for the first time that proline tRNA 3′ end maturation in *Escherichia coli* employs a one-step endonucleolytic pathway that does not involve any of the six 3′ → 5′ exonucleases (RNase T, RNase PH, RNase D, RNase BN, RNase II and polynucleotide phosphorylase [PNPase]) to generate the mature CCA terminus. Rather, RNase E is primarily responsible for the endonucleolytic removal of the entire Rho-independent transcription terminator associated with the *proK, proL* and *proM* primary transcripts by cleaving immediately downstream of the CCA determinant. In the absence of RNase E, RNase G and RNase Z are weakly able to process the *proK* and *proM* transcripts, while PNPase and RNase P are utilized in the processing of *proL*. The terminator fragment derived from the endonucleolytic cleavage of *proL* transcript is degraded through a PNPase-dependent pathway. It is not clear which enzymes degrade the *proK* and *proM* terminator fragments. Our data also suggest that the mature 5′ nucleotide of the proline tRNAs may be responsible for the cleavage specificity of RNase E at the 3′ terminus.

INTRODUCTION

Transfer RNAs (tRNAs) are essential components of the translation machinery in all domains of life. In *Escherichia coli* the 86 tRNA genes are transcribed either as polycistronic (containing other tRNAs or mRNAs or rRNAs) or monocistronic transcripts (1). Each of the primary transcripts undergoes extensive processing at both the 5′ and 3′ ends to generate a mature tRNA that can be aminoacylated. RNase E, an endoribonuclease, plays an important role in *E. coli* tRNA processing in some cases by either separating the individual pre-tRNAs from polycistronic tRNA transcripts or by removing Rho-independent transcription terminators, normally 1–3 nt downstream of the encoded CCA determinants to generate the substrates for the various 3′ → 5′ exonucleases (2–4). RNase P, the only endoribonuclease implicated in the 5′ end maturation of tRNAs in all kingdoms of life, generates the mature 5′ termini (5–7).

In contrast, the final maturation of the 3′ terminus of each tRNA is much more complex involving up to six 3′ → 5′ exonucleases: RNase T; RNase PH; RNase D; RNase BN; RNase II; and polynucleotide phosphorylase (PNPase) (8,9). RNase T is the major tRNA 3′ end processing enzyme unless there are C nucleotides immediately downstream of CCA determinant (10). Thus, with the *valU* pre-tRNA, RNase PH is the primary 3′ → 5′ processing enzyme (11). In the absence of both RNase T and RNase PH, a combination of RNase D, RNase BN/Z and RNase II can inefficiently complete 3′ end maturation (12). RNase BN, also called RNase Z, has both endo- and exonucleolytic activity *in vivo* (13–15). RNase II, which is normally involved in mRNA decay (16,17), and PNPase, which can also function as a poly(A) polymerase (18), play a role in 3′ end maturation under certain circumstances (9). In addition, poly(A) polymerase I (PAP I) has recently been shown to help regulate mature 3′ terminus formation (19,20).

In our analysis of the interaction of RNase T, RNase PH and poly(A) polymerase I (PAP I) in the generation of functional tRNAs, we observed that none of these enzymes were involved in the maturation of the three proline tRNAs (19). Furthermore, while all the mature *E. coli* tRNAs were resistant to polyadenylation by PAP I, most pre-tRNAs (79/86) were subject to extensive polyadenylation (19). In contrast, the three proline tRNAs were not polyadenylated under these conditions (19,20). Accordingly, we hypothesized that their mature 3′ ends were being generated by endonucleolytic cleavages immediately downstream of the CCA determinant, thereby preventing polyadenylation by PAP I.

The three $tRNAs^{Pro}$ genes (*proK, proL* and *proM*) in the *E. coli* genome recognize four different codons (CCC, CCG, CCA and CCU). While *proM* is transcribed as part of the *argX* polycistronic operon (*argX hisR leuT proM*) (2,3), *proK* and *proL* are believed to be transcribed as monocistronic transcripts (21) terminated with Rho-independent

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transcription terminators. The *proM* pre-tRNA is released from the *argX* polycistronic primary transcript by RNase E cleavages 3–4 nt upstream of its mature 5′ terminus (2,3). However, the exact mechanism for the removal of the Rho-independent transcription terminator at the 3′ end of *proM* and its final maturation are unknown. The processing pathways for the *proK* and *proL* primary transcripts have not been studied at all.

Here, we show that both *proK* and *proL* are transcribed as monocistronic transcripts terminated with Rho-independent transcription terminators. The 5′ ends of *proK* and *proL* are directly matured by RNase P cleavages. In contrast, the *proM* pre-tRNA is first separated from the *argX* polycistronic transcript by RNase E cleavages at 3, 4 and 15 nt upstream of the mature 5′ end. All three proline transcripts are matured at their 3′ ends by single RNase E endonucleolytic cleavages within the AU rich regions that are immediately downstream of the CCA, completely removing each Rho-independent transcription terminator. In the case of *proK* and *proM*, both RNase G and RNase Z can weakly substitute for RNase E. In contrast, the 3′ → 5′ exonuclease PNPase appears to be very effective in the 3′ end maturation of *proL* in the absence of RNase E. In addition, the endonuclease RNase P can weakly substitute for RNase E. Taken together, proline tRNA maturation is distinct from all previously described tRNA processing pathways in *E. coli*.

MATERIALS AND METHODS

Bacterial strains

The *E. coli* strains used in this study (Supplementary Table S1) were derived from MG1693, a *thyA715* derivative of MG1655 that was originally sequenced by Blattner *et al.* (1). Since all MG1655 derivatives contain a single base pair deletion in the *rph* gene that functionally inactivates RNase PH (*rph-1*) (22), we constructed an *rph*$^+$ derivative by P1 transduction to generate a wild-type control [SK10153, (19)]. SK5665 (*rne-1 rph-1*) (23), SK2525 (*rnpA49 rbsD296*::Tn*10 rph-1*) (3) and SK2534 (*rne-1 rnpA49 rbsD296*::Tn*10 rph-1*) (3) have been previously described. The *rne-1* and *rnpA49* alleles encode temperature sensitive RNase E and RNase P proteins, respectively, that do not support cell viability at 44°C (24–26). JW1644, JW5808 and JW1793 (27) were used as the donor strains for P1 transductions to delete the *rnt, pcnB* and *rnd* alleles, respectively. To facilitate the construction of multiple mutants, the FRT-flanked antibiotic resistance gene (Km^R) used for selection was removed using the FLP helper plasmid (pCP20) as described previously (28).

SK10625 (*Δrnd-729 rph-1*) was constructed by transduction of MG1693 using a phage P1 lysate grown on JW1793, followed by removal of the Km^R cassette. SK10629 (*Δrnd-729 Δrnz::apr rph-1*) was generated by transducing SK10625 with a P1 lysate grown on SK4478 (*Δrnz::apr rph-1*). A P1 lysate grown on SK10148 (*Δrnt::kan rph-1*) (3) was used to transduce SK10629 to generate SK10636 (*Δrnt::kan Δrnd-729 Δrnz::apr rph-1*).

SK10686 [*Δrnt::kan Δrnd-729 Δrnz::apr Δrnb::*Tn*10 rph-1*/pDK39 (*rnb-500*/Cm^R)] was constructed by plasmid displacement (29). Initially, SK10636 (*Δrnt::kan Δrnd-729 Δrnz::apr rph-1*) was transformed with pBMK62 (*rph*/Ap^R), which was derived by subcloning an EcoRI-BamHI fragment from pBMK61 (*rph*$^+$/Ap^R) (20) into pBR322 at the same sites. The resulting strain was used as a recipient for a transduction using a P1 lysate grown on CMA201 (*Δrnb::*Tn*10 rph-1*) (30) to generate SK10685 (*Δrnt*::*kan Δrnd-729 Δrnz*::*apr Δrnb*::Tn*10 rph-1*/pBMK62). Subsequently, pBMK62 was displaced with the plasmid pDK39 (*rnb-500*/Cm^R) (16), which has the same origin of DNA replication but carries the temperature sensitive *rnb-500* allele and selecting for Cm^R. SK10934 [*Δrnt::kan Δrnd-729 Δrnz::apr Δpnp-683 Δrnb::*Tn*10 rph-1*/pDK39 (*rnb-500*/Cm^R)] and SK10961 (*rne-1 Δpnp-683 rph-1*) were constructed by transducing SK10686 and SK5665, respectively, with P1 grown on SK10019 (*Δpnp-683 rph-1*). SK10953 (*rne-1ΔrhlB rph-1*) was constructed by transducing SK5665 with P1 grown on SK10553 (*ΔrhlB rph-1*) (9).

Growth of bacterial strains, isolation of total RNA and northern analysis

Unless noted otherwise in the text, all the bacterial strains were initially grown at 37°C in Luria broth (supplemented with 50 μg/ml thymine and appropriate antibiotics) with vigorous shaking until they reached 50 Klett units (No. 42 green filter) above background and were then shifted to 44°C for 60 min to inactivate RNase E (*rne-1*), RNase P (*rnpA49*) or RNase II (*rnb-500*). The cultures were maintained in exponential growth by periodically diluting the cultures with fresh Luria broth kept at 44°C.

Total RNA was extracted as described previously (31) and treated with DNase I using the DNA-free^{TM} kit (Ambion) to remove any residual DNA contamination. To facilitate equal loading, all RNAs were initially quantified by measuring the OD_{260} using a Nanodrop (ND2000c) apparatus. Subsequently, the RNA samples (500 ng) used for northern and primer extension analysis were normalized by quantifying Vistra Green (Amersham Bioscience) stained 16S and 23S rRNAs in agarose mini gels using a PhosphorImager (Storm 840, Amersham Bioscience). Total RNA was separated in either 6 or 10% polyacrylamide gels (noted in the figure legends) containing 8M urea in TBE [Tris-Borate-EDTA buffer] as described previously (4,32). All northern analyses were repeated at least three times. The quantification data (relative fold increase or decrease) mentioned in the text represent an average of three independent determinations.

Primer extensions

Primer extension analysis of the proline tRNA transcripts was carried out as described previously (32) with the following modifications. The *proK* and *proL* nucleotide sequences were obtained using the Thermo Sequenase Cycle Sequencing Kit (Affymetrix) and polymerase chain reaction (PCR) amplified DNA from wild-type genomic DNA, using primers upstream and downstream of the respective genomic sequences as templates. Primers a (Figure 1A) and c (Figure 2A) were used for both the reverse transcription and sequencing of *proK* and *proL*, respectively. The se-

quences were analyzed on 6% polyacrylamide sequencing gels (PAGE) containing 8M urea.

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

The 5′ and 3′ ends of proline tRNA transcripts were identified by cloning and sequencing the reverse transcription-PCR products obtained from 5′ → 3′ end-ligated circular RNAs following the methods described previously (31). The self-ligated proline tRNA transcripts were reverse transcribed using a mixture (1:1:1) of tRNA specific primers (PROK-S-CLA for *proK,* PROL-S-CLA for *proL* and PROM-S-CLA for *proM*) containing ClaI restriction site at the 5′ end. The 5′-3′ junctions of the resulting cDNAs were amplified in GoTaq®Green Master Mix (Promega) using the tRNA specific 3′-primer as noted above and a tRNA specific 5′ primer (PROK-SAC for *proK,* PROL-SAC for *proL* and PROM-SAC for *proM*). All the 5′ primers contained a SacII restriction site. The PCR products were cloned into pWSK29 (33) at SacII–ClaI sites and sequenced by Macrogen USA.

Oligonucleotide probes and primer selection

Since the nucleotide sequences for all three mature proline tRNAs are very homologous, probes and primers distinguishing each of the tRNA were designed based on a previous report (34). The oligonucleotides used either as probes during Northern hybridizations or primers for primer extension, reverse transcription and PCR amplifications are shown in Supplementary Table S2.

RESULTS

The *proK* and *proL* primary transcripts have short (5–7 nt) 5′ leader sequences

Although the *proK* and *proL* genes are believed to be transcribed as monocistronic transcripts with 2–4 nt leader sequences (21), neither of the actual transcription start-sites have been determined. Accordingly, we mapped their transcription start-sites using primer extension analysis of RNA isolated from RNase PH deficient (*rph-1*) and temperature sensitive RNase E and RNase P multiple mutants *(rne-1 rph-1* and *rnpA49 rph-1*). For *proK*, a single primer extension product representing the mature 5′ terminus (M, Supplementary Figure S1A) was observed in the *rph-1* strain (lane 5). The intensity of this band was reduced in the *rne-1 rph-1* strain (lane 6) and remained almost unchanged in an *rnpA49 rph-1* double mutant (lane 7). The primer extension products M + 5 and M + 6 observed in the *rne-1 rph-1* strain increased in their intensities in the *rnpA49 rph-1* strain and were absent in the *rph-1* strain.

For *proL*, a major primer extension product (M, Supplementary Figure S1B) was observed at the annotated mature 5′ end in both the *rph-1* and *rne-1 rph-1* strains, but was significantly reduced in the *rnpA49 rph-1* strain. Additional sequencing experiments confirmed this to be the mature 5′ end of *proL* (see below). However, a minor band M+1 with reduced intensity compared to M was also observed one nucleotide upstream of the mature 5′ end in all the strains. The dependence of both M and M + 1 on RNase P cleavage suggested a primary and a secondary RNase P cleavage site, respectively, for *proL*. Unlike with *proK*, additional primer extension products (M + 6, M + 7 and ∗) were observed in all genetic backgrounds. The level of bands M + 6 and M + 7 at 6 and 7 nt, respectively, upstream of the 5′ mature end (M) was slightly higher in the *rne-1 rph-1* strain compared to the *rph-1* strain and increased significantly in the *rnpA49 rph-1* strain. The band marked with a star (∗) was probably a non-specific primer extension product, since its level did not change in any genetic background (Supplementary Figure S1B). This conclusion was supported by the fact that no higher molecular weight transcripts were observed during northern analysis and RT-PCR cloning experiments (see below).

It should be noted that the bands M + 5 and M + 6 for *proK* and M + 6 and M + 7 for *proL* were stabilized in the absence of RNase P (Supplementary Figure S1A and B, lane 7), the only endoribonuclease known to generate the mature 5′ end of any tRNA (5–7). The existence of small amounts of these products in the RNase E deficient strain (Supplementary Figure S1A and B, lane 6) may have resulted from inefficient RNase P cleavage at the 5′ end due to presence of the Rho-independent transcription terminator at the 3′ end (4) (see also below). Thus, M + 5 and M + 6 were considered to be the transcription initiation sites of the *proK* primary transcripts and M + 6 and M + 7 were considered to be the transcription initiation sites of the *proL* primary transcripts (Supplementary Figure S1C and D). These data were consistent with identification of a consensus −10 (4/6 nt) and −35 sequences upstream of both *proK* and *proL* transcription initiation sites (Supplementary Figure S1C and D). Furthermore, cloning and sequencing data of cDNAs obtained from tobacco ccid pyrophosphatase treated *proK* and *proL* transcripts supported this conclusion (see 'Discussion' section).

Maturation of *proK*, *proL* and *proM* tRNA 3′ ends is independent of 3′→ 5′ exoribonucleases

To date, the final 3′ end maturation of all tRNAs studied in *E. coli* has been shown to be dependent on at least one or a combination of six different 3′ → 5′ exoribonucleases including RNase T, RNase PH, RNase BN, RNase D, RNase II and PNPase, irrespective of the initial 3′ end cleavage (9,35,36). For most tRNAs, the absence of just RNase T and RNase PH leads to the accumulation of tRNA species that retain 1–3 nt immediately downstream of their CCA determinants (19). In order to understand if the 3′ end maturation of the proline tRNA primary transcripts followed a similar pathway, we analyzed various strains deficient in up to six of the known exoribonucleases (RNase T, RNase PH, RNase BN, RNase D, RNase II and PNPase) involved in tRNA 3′-end processing, employing Northern analysis and probes specific for either the mature tRNA or the Rho-independent transcription terminator.

A probe specific to the mature *proK* species (probe a, Figure 1A) showed no effect on the processing of the *proK* tRNA in the absence of all six exoribonucleases (Figure 1B, lanes 1–5). A single band, representing the mature tRNA, was observed in all the strains. A probe specific to the *proK*

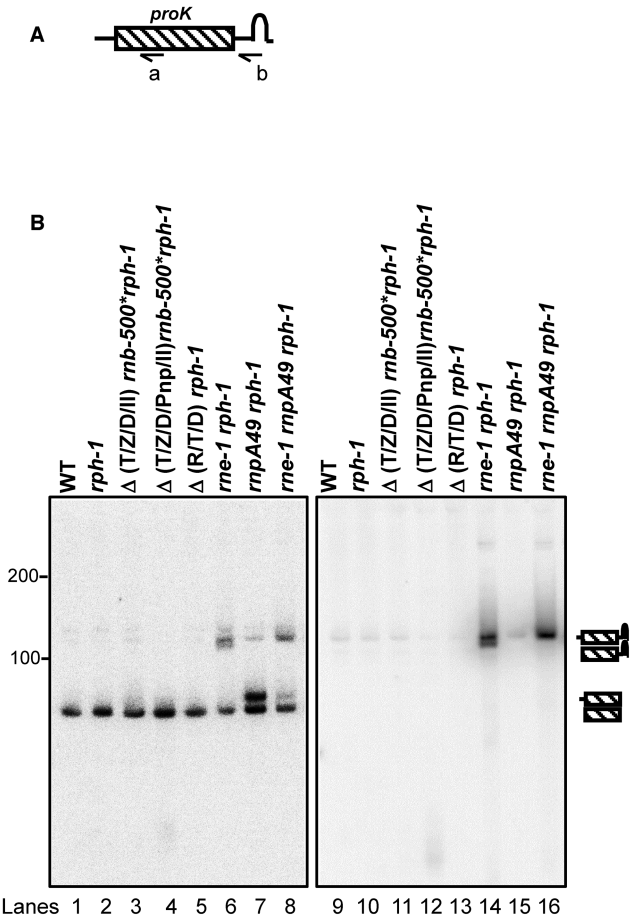

Figure 1. Northern analysis of *proK* transcripts in various genetic backgrounds. (**A**) Schematic representation of *proK* gene (not drawn to scale). Relative position of the oligonucleotide probes (a, PROK-2 and b, PROK-TER) used in the northern analysis are shown below the cartoon. (**B**) A representative northern blot analysis of *proK* transcript. Total RNA (15 μg/lane) was separated on 10% PAGE, transferred to a nylon membrane and probed sequentially with probes a (lanes 1–8) and b (lanes 9–16) as described in 'Materials and Methods' section. The deduced structures of the processing intermediates of the *proK* transcripts are shown to the right of the blot. The genotypes of the strains used are indicated above each lane. Δ(T,Z,D, II and Pnp) represent chromosomal deletion/substitution mutants for the enzymes RNase T, RNase Z/BN, RNase D, RNase II and polynucleotide phosphorylase (PNPase), respectively. *rne-1, rnpA49* and *rnb-500* encode temperature sensitive RNase E, RNase P and RNase II proteins, respectively (see 'Materials and Methods' section). The genotype marked with an asterisk (*) denotes that the deleted chromosomal *rnb* gene is complemented by the temperature sensitive *rnb-500* allele (pDK39). The RNA size standards (nucleotides, Fermentas) are shown to the left of the blot.

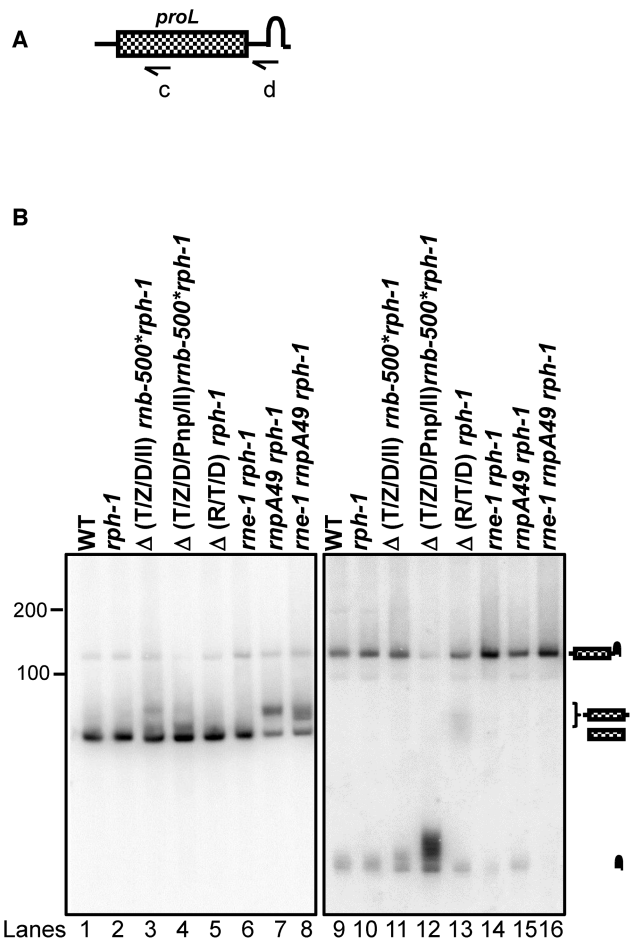

Figure 2. Northern analysis of *proL* transcripts in various genetic backgrounds. (**A**) Schematic representation of *proL* gene (not drawn to scale). Relative positions of the oligonucleotide probes (c, PROL-2 and d, PROL-TER) used in the northern analysis are shown below the cartoon. (**B**) A representative Northern blot analysis of *proL* transcript using probes c (lanes1-8) and d (lanes 9–16). The experiment was carried out as described in Figure 1. The deduced structures of the processing intermediates of *proL* transcripts are shown to the right of the blot. The genotypes of the strains used are indicated above each lane. Δ(T,Z,D, II and Pnp) represent chromosomal deletion/substitution mutants for the enzymes RNase T, RNase Z/BN, RNase D, RNase II and polynucleotide phosphorylase (PNPase), respectively. *rne-1, rnpA49* and *rnb-500* encode temperature sensitive RNase E, RNase P and RNase II proteins, respectively (see 'Materials and Methods' section). The genotype marked with an asterisk (*) denotes that the deleted chromosomal *rnb* gene is complemented by the temperature sensitive *rnb-500* allele (pDK39). The size standards were the same as used in Figure 1.

transcription terminator region (probe b, Figure 1A) hybridized very weakly to a larger species of ∼113 nt, the predicted size of a full-length primary transcript that retained the Rho-independent transcription terminator (lanes 9–13), suggesting that the *proK* primary transcripts were processed efficiently in all genetic backgrounds. The *proK* terminator probe (b) also hybridized very weakly to a 30–35 nt heterogeneous band in the sextuple mutant (lane 12), which was predicted to be the partial or full-length *proK* terminator.

A mature *proL* specific probe (probe c, Figure 2A) hybridized to the mature species of ∼77 nt, which was the most prominent band in all the exonuclease deficient strains (Figure 2B, lanes 1–4). In addition to the mature species, a faint band at ∼120 nt, predicted to be the full-length primary transcript containing the Rho-independent transcription terminator, was also present in all the strains including the wild-type control. Importantly, no significant difference in the size of the mature tRNA was observed among the strains deficient in up to six exonucleases (Figure 2B, lanes 1–4, data not shown). The few higher molecular weight species observed in the multiple exunuclease mutants (lanes 3–4) were likely due to polyadenylation, since they disap-

peared in the corresponding *pcnB* deletion strains (data not shown, see below).

A probe complementary to the transcription terminator region (probe d, Figure 2A) hybridized to both the ∼120 nt species confirming it to be the full-length transcript (Figure 2B, lanes 9–13) and to the highly heterogeneous smaller species of ∼40–50 nt in all strains (Figure 2B, lanes 9–13). The smaller species were identified as the full-length terminator or part of it based on hybridization to a second probe downstream of the probe d (data not shown). The level of both full-length and the terminator bands remained identical in all the strains except the sextuple mutant (lane 12) where the amount of the terminator species increased ∼5 ± 2-fold and the level of the full-length species decreased ∼50%. The presence of the full-length species in all the strains including the wild-type control suggested that the *proL* transcript was not processed efficiently.

Unlike *proK* and *proL, proM* is transcribed as part of a polycistronic operon (*argX hisR leuT proM*). RNase E plays a major role in separation of individual pre-tRNAs from the primary transcript (2,3). However, nothing is known regarding the removal of the downstream Rho-independent transcription terminator. The 3′ end maturation of *proM* was analyzed using a probe (e) specific to the mature *proM* species (Figure 3A). No significant accumulation of species 1–3 nts larger than the mature tRNA was observed even in the sextuple mutant, suggesting no major role for any of the 3′→ 5′ exonucleases (Figure 3, lanes 1–5).

If any of the 3′ exonucleases were involved in processing the proline tRNAs, a band one–two nucleotides larger than fully mature species would have been observed in the 10% polyacrylamide gels (Figures 1–3), as was observed when we reprobed the membrane for either the *leuX*, *pheU* and *pheV* tRNAs (Supplementary Figure S2). These three tRNAs have been shown to utilize both RNase T and RNase PH for their final 3′ end maturation (9,19). As shown in Supplementary Figure S2, pre-tRNAs that were 1–2 nt larger than the mature tRNAs accumulated for all three tRNAs in the absence of the various 3′ → 5′ exonucleases. This result was distinct from what was observed for the three proline tRNAs (Figures 1–3).

In addition to the six exoribonucleases tested above, *E. coli* contains a seventh 3′ → 5′ exonuclease called RNase R, which is not inhibited by RNA secondary structure (37), requires a 7 nt single-stranded region for substrate binding (38), but has not been implicated in *E. coli* tRNA maturation. Even so, we investigated its potential involvement in the 3′ end processing of *proK, proL* and *proM* tRNAs, since it has been shown to participate in tRNA 3′ end processing in *Mycoplasma genitalium* (39). However, no effect of RNase R was observed in an *Δrnr Δrnt Δrnd rph-1* multiple mutant (Figures 1 and 2, lanes 5 and 13; Figure 3, lane 5).

Maturation of *proK*, *proL* and *proM* tRNA 3′ ends is highly dependent on endonucleolytic cleavages

Since none of the 3′ → 5′ exonucleases had any significant effect in the 3′ end maturation of *proK, proL* and *proM* (Figures 1–3), we investigated their processing in strains containing thermosensitive RNase E (*rne-1*) and/or

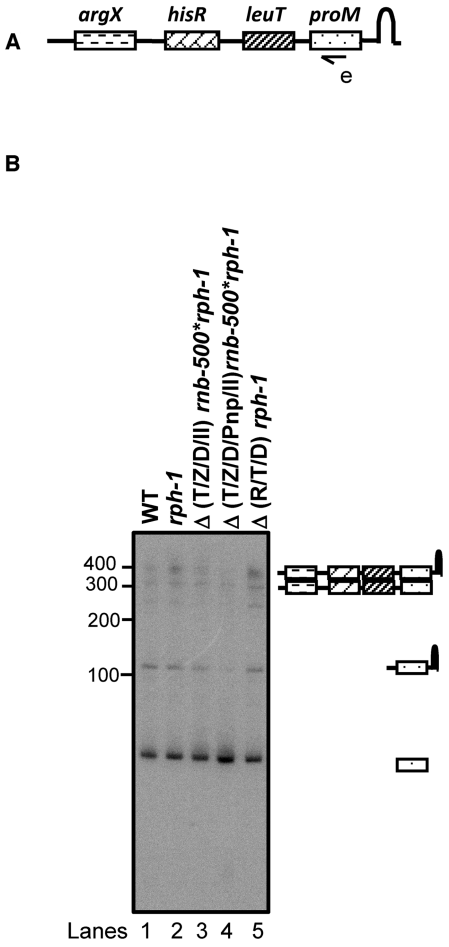

Figure 3. Northern analysis of *proM* transcripts in various genetic backgrounds. (**A**) Schematic representation of *argX* operon (not drawn to scale). Relative position of the oligonucleotide probe (e, PROM-S2) used in the northern analysis are shown below the diagram. (**B**) Representative Northern blot analysis of *proM* transcript using probe e. The experiment was carried out as described in Figure 1. The deduced structures of the processing intermediates of the *proM* transcripts are shown to the right of the blot. Δ(T,Z,D, II and Pnp) represent chromosomal deletion/substitution mutants for the enzymes RNase T, RNase Z/BN, RNase D, RNase II and polynucleotide phosphorylase (PNPase), respectively. The *rnb-500* allele encodes a temperature sensitive RNase II protein (see 'Materials and Methods' section). The genotypes marked with an asterisk (*) denote that the deleted chromosomal *rnb* gene is complemented by *rnb-500* allele (pDK39). The size standards were the same as used in Figure 1.

RNase P (*rnpA49*) proteins at the non-permissive temperature (44°C). In the case of *proK*, probe a hybridized to the mature as well as the ∼113 nt species in the *rne-1 rph-1, rnpA49 rph-1* and *rne-1 rnpA49 rph-1* strains (Figure 1B, lanes 6–8). The largest species of ∼113 nt was confirmed to be the full-length primary transcript that retained the Rho-independent transcription terminator based on its hybridization to both probes a and b (Figure 1B, lanes 14–16). The level of the *proK* full- length transcripts increased ∼15-fold in the *rne-1 rph-1* and *rne-1 rnpA49 rph-1* strains (Figure 1, lanes 6, 8, 14 and 16) compared to the wild-type control. Probe b also hybridized to the second largest band in the *rne-1 rph-1* strain (Figure 1B, lanes 6 and 14) indicating that it retained the Rho-independent transcription termina-

tor, but may have lost some sequences at the 5′ terminus (see below).

In the *rnpA49 rph-1* mutant, in addition to the mature tRNA, a new species of 5–6 nt larger than the mature tRNA was readily identified by probe a (Figure 1B, lane 7). The significantly reduced amount of the larger 5–6 nt species in the *rne-1 rnpA49 rph-1* triple mutant (Figure 1B, lane 8) compared to the *rnpA49 rph-1* double mutant (lane 7) suggested that these species arose from the retention of the primary 5′ terminus in the absence of RNase P and removal of the Rho-independent transcription terminator by RNase E. The presence of the higher level of full-length transcripts in the *rnpA49 rph-1* mutant compared to the wild-type control strain also indicated that RNase E removal of the Rho-independent transcription terminator was partially inhibited by the presence of the 5′ terminus (Figure 1B, lanes 1 and 7).

In the case of *proL,* as expected, both the mature and the full-length species hybridized to probe c in the *rne-1 rph-1, rnpA49 rph-1* and *rne-1 rnpA49 rph-1* mutants (Figure 2B, lanes 6–8). Probe d hybridized to the full-length and the terminator species (Figure 2B, lanes 14–15). Based on the results with *proK* (Figure 1B, lanes 6 and 14), it was surprising that the inactivation of RNase E at 44°C only led to a small increase (∼2.5 ± 0.5-fold) in the level of the full-length *proL* species compared to the wild-type control (Figure 2B, lanes 1, 6, 9 and 14). The inactivation of RNase P led to the reduction of ∼3 ± 0.5-fold in the mature tRNA species with a concomitant accumulation of several processing intermediates above the mature species (Figure 2B, lanes 1 and 7). These species did not contain the Rho-independent transcription terminator, since they did not hybridize to probe d (lanes 15 and 16), but probably retained the unprocessed 5′ end in the absence of RNase P.

The amount of the terminator fragment detected by probe d was reduced ∼50% in the *rne-1 rph-1* double mutant compared to the wild-type strain and disappeared completely in the *rne-1 rnpA-49 rph-1* triple mutant (Figure 2B, lanes 9, 14 and 16), suggesting that the terminator was primarily removed by RNase E. Thus, RNase P acted as a backup enzyme to RNase E, since the level of the terminator fragment and full-length species in the *rnpA49 rph-1* double mutant remained almost identical to the wild-type strain (lanes 9 and 15).

It has been previously shown that RNase E removes the *proM* pre-tRNA from the *argX* operon by cleaving 3–4 nt upstream of *proM* 5′ mature end (2,3). Accordingly, inactivation of RNase E led to an approximately 5.3 +/- 0.5-fold increase in unprocessed *proM* species (Supplementary Figure S3c, lane 2).

Endoribonucleases other than RNase E and RNase P contribute to the 3′ maturation of the proline tRNAs

The data presented above (Figures 1 and 2; Supplementary Figure S3C), as well as work published earlier (2,3), suggested that RNase E was involved in the processing of all three proline primary transcripts. However, the inactivation of RNase E more dramatically affected the processing of *proK* and *proM* compared to *proL.* Thus, not only was there an increase in the amount of the full-length *proK* (∼15 ± 3.5-fold) and *proM* (∼5.3 ± 0.5-fold) species, their mature tRNA levels were also reduced ∼40–50% in the absence of RNase E (Figure 1B, lanes 6 and 14; data not shown) (3). In the case of *proL*, although there was ∼2.5 ± 0.5-fold increase in the level of the *proL* primary transcript in the absence of RNase E, the levels of mature *proL* tRNA in both wild-type and *rne-1 rph-1* strains (Figure 2B, lanes 1 and 6) were similar. In addition, the level of full-length transcripts in the *rne-1 rph-1* and *rne-1 rnpA49 rph-1* was also similar (lanes 14 and 16), suggesting that the processing of the primary *proL* transcript was being carried out by other ribonucleases.

Besides RNase E, *E. coli* contains RNase G, RNase Z and RNase LS as potential contributors to tRNA processing. In fact, RNase G and RNase Z have previously been shown to be involved in tRNA processing (4,40). RNase LS, encoded by *rnlA,* has been shown to be responsible for degradation of various mRNAs in *E. coli* (41). Accordingly, the steady-state level of each of the three proline tRNAs was analyzed by northern analysis using probes specific to each of the mature species in mutant strains inactivated for various combinations of RNase E, RNase G, RNase Z and RNase LS (Supplementary Figure S3). As expected, the unprocessed *proK* transcripts increased ∼15-fold in the *rne-1 rph-1* double mutant compared to the *rph-1* control (Supplementary Figure S3A, lanes 1–2). The level of the full-length *proK* transcript increased further (∼24-fold) in the *rne-1 Δrng Δrnz ΔrnlA rph-1* multiple mutant compared to the *rph-1* strain (Supplementary Figure S3A, lanes 1 and 3), suggesting that either RNase G, RNase Z or RNase LS might also be involved in the removal of its Rho-independent transcription terminator. The level of the *proK* unprocessed transcripts in the *rne-1 Δrng Δrnz rph-1* mutant was similar to the *rne-1 Δrng Δrnz ΔrnlA rph-1* mutant (Supplementary Figure S3A, lanes 3–4), but decreased to the *rne-1 rph-1* level in the *rne-1 Δrng ΔrnlA rph-1* mutant (Supplementary Figure S3A, lanes 2 and 5) and *rne-1 Δrnz ΔrnlA rph-1* (data not shown), ruling out RNase LS in *proK* tRNA processing. Consistent with the increase in the level of the full-length transcripts, the relative amount of the mature *proK* tRNA was also reduced ∼40–60% in the *rne-1 Δrng Δrnz ΔrnlA rph-1* and *rne-1 Δrng Δrnz rph-1* mutants (Supplementary Figure S3A, lanes 1–5).

For *proL*, the level of the full-length transcript in the *rne-1 rph-1* double mutant and *rne-1 Δrng Δrnz ΔrnlA rph-1* quintuple mutant only increased 3- and 4-fold, respectively, compared to the *rph-1* strain (Supplementary Figure S3B, lanes 1, 2 and 3). The amount of the full-length transcript in the other two multiple endonuclease mutants was similar to the *rne-1 rph-1* double mutant (Supplementary Figure S3B, lanes 2, 4 and 5). However, the increase in the level of the full-length transcripts in all the mutants had very little effect on the level of the mature *proL* tRNA, since it was comparable in all five strains (Supplementary Figure S3B). RNase III (*rnc*) and YbeY (*ybeY*), two other known endoribonucleases primarily involved in rRNA processing (42,43), were also ruled out by examining *proL* maturation in *rnc ybeY* multiple mutants (*rncΔ38 rph-1*, *rncΔ38 rne-1 rph-1*, *ΔybeY rph-1*, and *ΔybeY rne-1 rph-1*) (data not shown).

In the case of *proM*, the results were slightly more complicated because this species is part of the *argX* polycistronic

transcript that is initially processed by RNase E (2,3). Accordingly, we expected a dramatic change in the northern blot between the *rph-1* strain and any RNase E mutant. As seen in Supplementary Figure S3C, the level of unprocessed transcripts (full-length and additional processing intermediates, combined) remained similar in all the strains inactivated for RNase E (lanes 2–5). However, the level of the full-length transcripts increased ∼2-fold in the *rne-1 Δrng Δrnz ΔrnlA rph-1* and *rne-1 Δrng Δrnz rph-1* mutants (lanes 3–4) compared to *rne-1 rph-1* and *rne-1 Δrng ΔrnlA rph-1* mutants (lanes 2 and 5). These data again indicated that RNase LS was not involved in *proM* processing. However, the results suggested that both RNase G and RNase Z were weakly involved in the processing of both *proK* and *proM* in the absence of RNase E.

The *proL* tRNA primary transcript was processed by both RNase E and PNPase

The fact that the *proL* terminator fragment was significantly stabilized in the sextuple exonuclease mutant compared to the quintuple exonuclease mutant (Figure 2B, lanes 11–12) suggested that PNPase was the primary exoribonuclease responsible for degrading the terminator fragment generated by RNase E cleavage. Accordingly, we hypothesized that in the absence of RNase E, the *proL* tRNA primary transcript was most likely being processed by PNPase. In fact, the level of the full-length *proL* transcript increased ∼40 ± 5-fold in the *rne-1 Δpnp-683 rph-1* triple mutant compared to ∼2.5 ± 0.5-fold in the *rne-1 rph-1* strain (Figure 4, lanes 2–3 and 6–7). Consequently, there was ∼50% reduction in the mature tRNA levels in the *rne-1 Δpnp-683 rph-1* triple mutant (Figure 4, Lanes-2-3). Interestingly, the *proL* processing profile in an *rne-1 ΔrhlB rph-1* triple mutant was identical to an *rne-1 rph-1* double mutant (data not shown), suggesting that the RhlB RNA helicase is not required for *proL* terminator processing by PNPase.

A single RNase E cleavage downstream of CCA rather than 3′→ 5′ exonucleolytic processing generates the mature 3′ terminus in proline tRNAs

The northern analysis data presented in Figures 1–3 clearly suggested that the three proline tRNAs were matured independent of all the known 3′ → 5′ exoribonucleases. Furthermore, the data indicated that the endonucleolytic activities of RNase P at the 5′ end and RNase E at the 3′ end were primarily involved in the maturation of all three proline tRNAs. Although high resolution 10% polyacrylamide gel electrophoresis was used to distinguish between mature and immature species, tRNAs with similar sizes but different nucleotides at either their 5′ or 3′ ends cannot be readily distinguished by this technique. Thus, nucleotide sequences at both the 5′ and 3′ ends of all three proline tRNAs were specifically determined by sequencing cDNAs generated by reverse transcription of self-ligated tRNAs from *rph-1*, *Δrnt rph-1*, *ΔpcnB rph-1, rne-1 rph-1*, *rne-1 Δrng Δrnz ΔrnlA rph-1* and *Δrnt Δrnd Δpnp Δrnb rph-1*/ pDK39 (*rnb-500* Cm^r) strains. We predicted that a large majority of the proline tRNA 3′ ends in a *Δrnt rph-1* double mutant would be mature due to a direct RNase E cleavage downstream of CCA in contrast to what was observed for tRNA^{His} and tRNA^{Cys}, where RNase E cleaved 1–2 nt downstream of CCA and a significant proportion of these species contained unprocessed nucleotides including poly(A) tails (19,20).

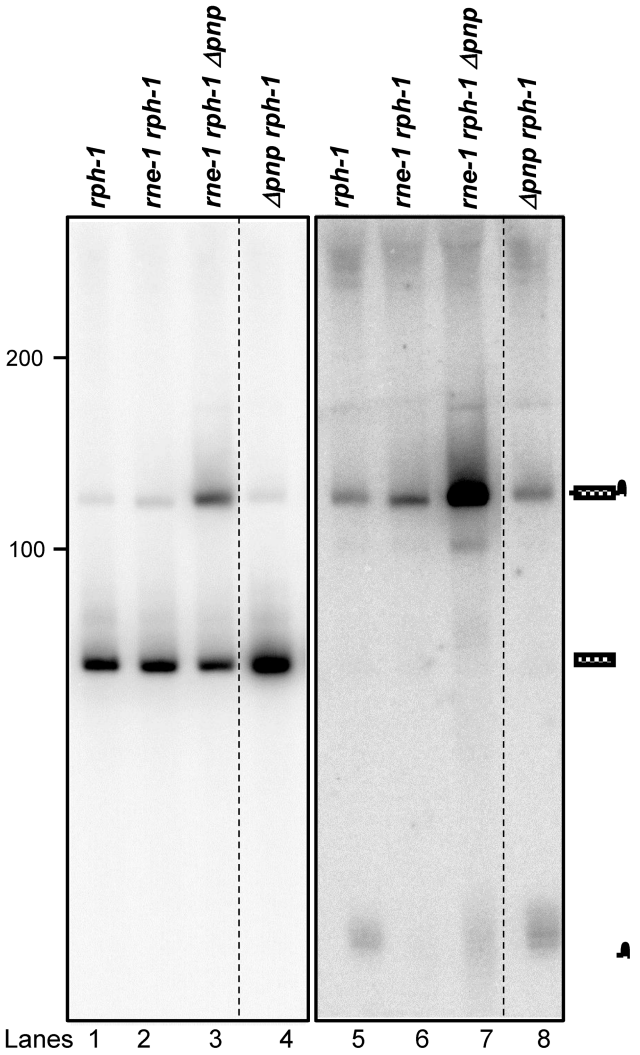

Figure 4. Northern analysis of *proL* transcripts in RNase E and PNPase mutants. Total RNA (15 μg/lane) was separated using a 10% PAGE, transferred to a nylon membrane and successively probed with probes specific to either *proL* mature species (probe c, Figure 2A) (lanes 1–4) or *proL* terminator species (probe d, Figure 2A) (lanes 5–8). The genotypes of the strains used are indicated at the top of each lane. ΔPnp is a deletion/substitution mutation (*pnpΔ683*) of PNPase. The deduced structures of the processing intermediates of all tRNA transcripts are shown to the right of the blot. The difference in the hybridization intensities of the full length *proL* transcripts in the *rne-1 rph-1* ΔPnp strain in lanes 3 and 7 were most likely due to difference in the specific activity and hybridizing efficiency of probes c and d. Lanes that were not relevant were removed from the blot (dashed line). The size standards were the same as used in Figure 1.

As shown in Figure 5 (A–C), only 12.5% (3/24) for each proline tRNA (*proK, proL* and *proM*) had 3′ immature sequences in the *rph-1* single mutant. The three immature *proM* clones retained the entire 3′ Rho-independent transcription terminator (Figure 5C, *), suggesting inefficient endonucleolytic processing. The percentage of 3′ immature sequences remained identical for *proK* tRNA (12.5%, 3/24

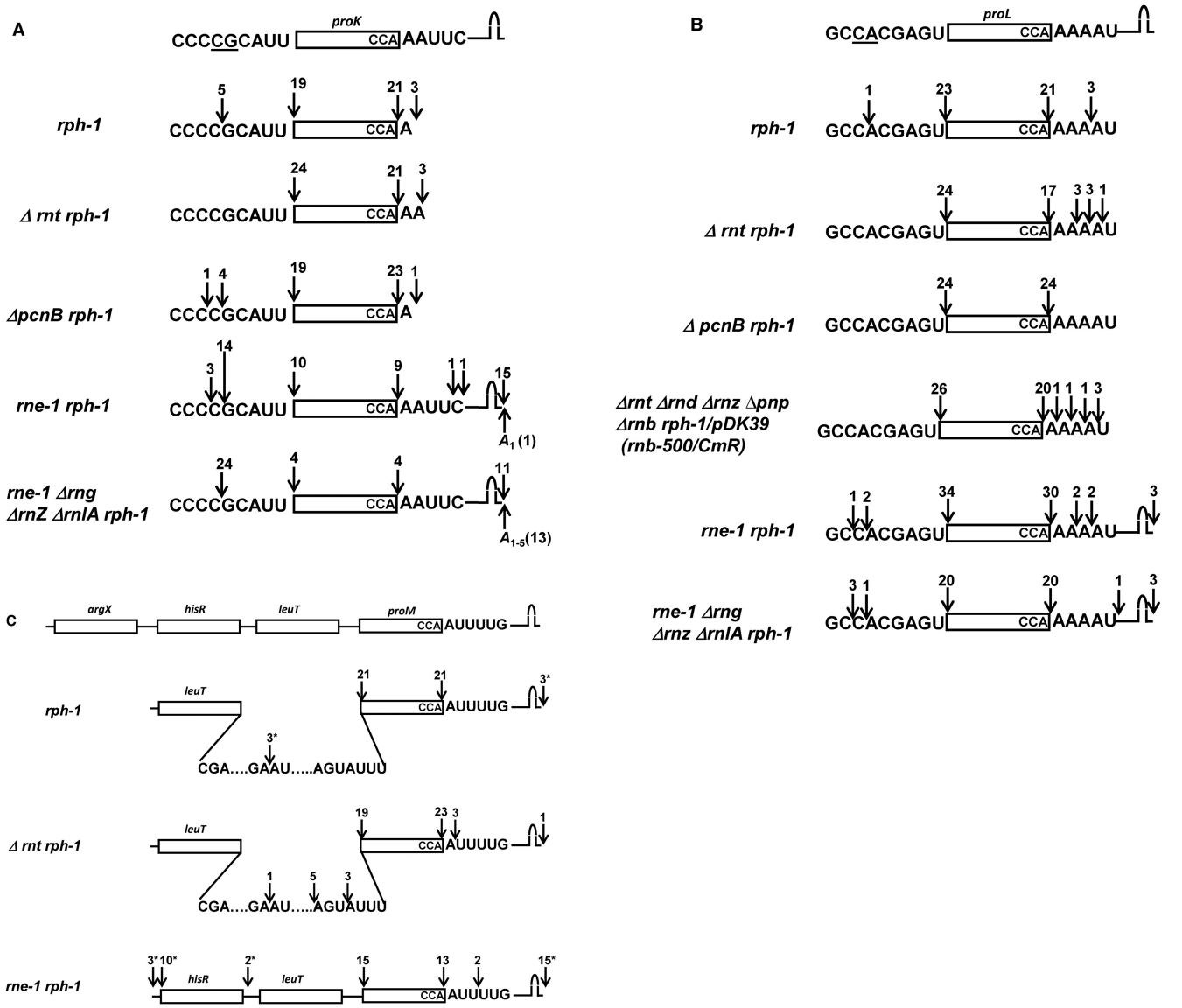

Figure 5. Determination of 5′ and 3′ termini of proline tRNAs using RT-PCR cloning of 5′-3′ self-ligated transcripts in various genetic backgrounds. (**A**) *proK*, (**B**) *proL* and (**C**) *proM*. The cartoon at the top of each panel is a graphic representation of the respective mature tRNA and the coding sequences upstream and downstream of the 5′ and 3′ ends, respectively. *proK* and *proL* transcription start sites (Supplementary Figure S1C and D) are underlined. Each downward arrow represents either a 5′ or 3′ end as determined by sequence analysis. Each upward arrow represents a 3′ end with untemplated poly(A) tail(s). (*) Sequences that retained their immature 5′ termini.

clones), while it increased to 29% (7/24) for *proL* and 15% (4/27) for *proM* in the *Δrnt rph-1* double mutant (Figure 5A–C). When we cloned and sequenced the proline tRNAs from a strain inactivated for RNase T, RNase D, RNase Z/BN, RNase PH and RNase II (*Δrnt Δrnd Δrnz Δrnb rnb-500 rph-1*), the sequencing profiles were experimentally identical (Supplementary Figure S4) to what was observed in the*Δrnt rph-1* double mutant (Figure 5A–C). Since PNPase had a significant effect on *proL* tRNA processing (Figures 2 and 4), we also cloned and sequenced *proL* cDNAs from the sextuple mutant (*Δrnt Δrnd Δrnz Δpnp-683 Δrnb rph-1*) (Figure 5B). The data showed that ∼77% (20/26) of tRNAs had the mature 3′ ends. The remaining clones (6/26) had 1–4 nts of A residues, some of which might have been added post-transcriptionally (see 'Discussion' section).

Significantly different sequencing profiles were observed in the *rne-1 rph-1* double mutant. Loss of RNase E led to 59% (16/27) of *proK* and 50% (15/30) of *proM* clones retaining the intact Rho-independent transcription terminator. Furthermore, all the *proM* clones with the Rho-independent transcription terminator (15/15) retained upstream tRNA sequences in the operon (Figure 5C, *). In contrast, only 8% (3/37) of *proL* clones retained the Rho-independent transcription terminator. We also sequenced the *proK* and *proL* tRNAs derived from the *rne-1 Δrng Δrnz ΔrnlA rph-1* strain. As expected, the level of the *proK* full-length transcripts increased significantly to ∼86% (24/28

clones) (Figure 5A). In contrast, the level of the *proL* full-length transcripts increased only to ∼12% (3/24 clones) (Figure 5B).

Thus the cloning data were consistent with the results obtained from the northern analysis (Figures 1–3). Furthermore, the very low percentage of immature 3′ ends of proline tRNAs in *Δrnt rph-1* mutant was consistent with direct endonucleolytic cleavage by RNase E downstream of CCA. These results were in contrast to what has been observed with the *hisR, cysT* and *leuX* tRNAs where ∼66% of the clones had immature sequences in the *rph-1* single mutant and increased to 100% in the *Δrnt rph-1* double mutant where RNase E cleaved 1–2 nt downstream of CCA (4,9,19,20). It is worth noting that the few *proK* and *proL* transcripts with extra As in the *rph-1, rph-1 Δrnt* and *rph-1 ΔpcnB* strains (Figure 5A and B) may have arisen from occasional RNase E cleavages 1–2 nt downstream of CCA and would be substrates for both 3′ → 5′ exonucleases and PAP I.

The full-length *proK* and 5′ unprocessed *proL* transcripts are polyadenylated under certain circumstances

Unlike most tRNAs, proline tRNAs are not substrates for polyadenylation in exonuclease deficient mutants (19), presumably because of the endonucleolytic processing that generates mature 3′ termini (Figures 1–3 and 5). Even overexpression of PAP I had little effect on any of the proline tRNAs in an *rph-1* single mutant (20). However, northern analyses showed that *proL* processing intermediates retaining their 5′ unprocessed ends in the *rnpA49 rph-1* double mutant had higher molecular weights compared to *proK* intermediates (Figures 1B and 2B, lane 7). The larger *proL* processing intermediates were inconsistent with retaining only the 6–7 nt unprocessed 5′ ends in an *rnpA49* mutant. The fact that the band of *proL* processing intermediates became significantly smaller in an *rnpA49 ΔpcnB rph-1* mutant strongly suggested that the majority of the species were polyadenylated in the *rnpA49 rph-1* strain (Figure 6A, lanes 3 and 4). This phenomenon was not observed with *proK* (Figure 6A, lanes 1 and 2).

Accordingly, we cloned and sequenced *proK* and *proL* tRNAs from *rnpA49 rph-1* and *rnpA49 ΔpcnB rph-1* genetic backgrounds (Figure 6B). As expected, ∼89% (24/27) of *proK* clones had 4–5 nt immature 5′ ends in *rnpA49 rph-1* double mutant. However, all 27 clones had mature 3′ ends. In the *rnpA49 ΔpcnB rph-1* triple mutant, ∼12% (4/34) of *proK* clones were full-length and overall ∼88% (30/34) of clones retained 5–6 nt immature 5′ ends. While ∼68% (23/34) of the clones had mature 3′ ends, ∼21% (7/34) of the clones had an 'A' downstream of CCA, most likely due to some processing at this site. It should be noted that although none of the partially processed *proK* clones in Figure 6B had untemplated poly(A) tails, ∼54% (13/24) of the full-length *proK* clones in *rne-1 Δrng ΔrnZ ΔrnlA rph-1* multiple mutants had poly(A) tails of 1–5 nts (Figure 5A).

In contrast to the *proK* results, ∼10% (3/31) of *proL* clones had at least one untemplated A in the *rnpA49 rph-1* mutant. In addition, ∼42% (13/31) of the clones had 1–5 nt 3′ As, which could have been added by PAP I. Approximately 58% (18/31) of the clones retained the 6–7 nt upstream 5′ end. Surprisingly, ∼89% (16/18) of the clones that retained the immature 5′ ends also had 3′ potential poly(A) tails. In the *rnpA49 ΔpcnB rph-1* triple mutant, ∼54% (14/26) of the *proL* clones had immature 5′ ends. However, none of the 3′ ends had either templated or untemplated 'A's suggesting that the As observed in the *rnpA49 rph-1* strain arose from polyadenylation. Moreover, 8% (2/26) of the clones were full-length and 12% (3/26) of the clones were processed 6 nt downstream of CCA and all had retained the immature 5′ end (Figure 6B, *). These sequencing data were consistent with the northern analysis (Figure 6A).

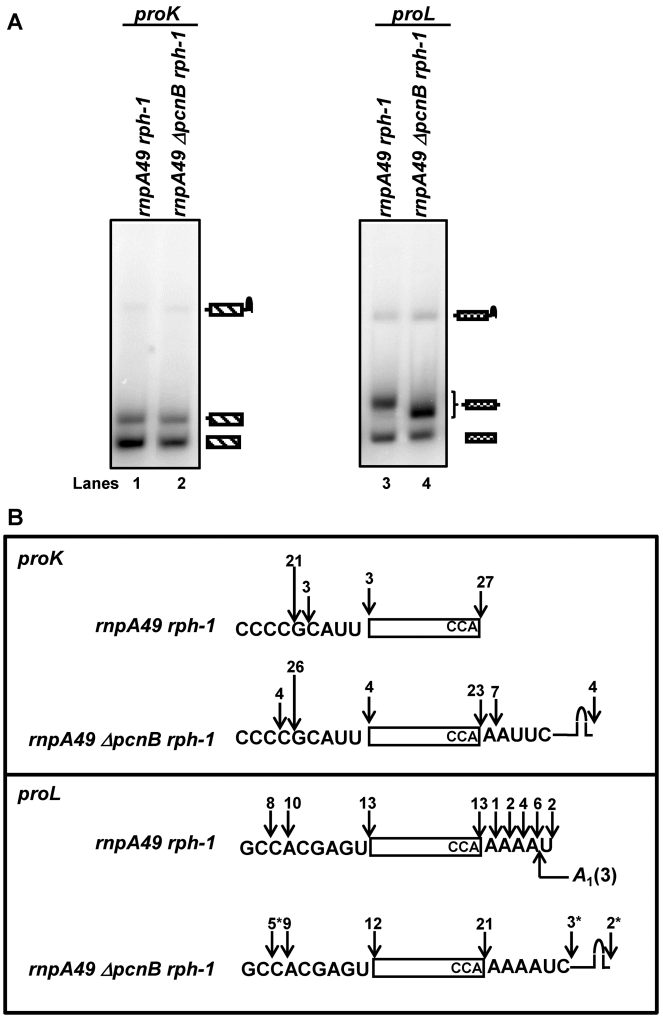

Figure 6. Analysis of polyadenylation of the *proK* and *proL* tRNAs in *rnpA49 rph-1*and *rnpA49 ΔpcnB rph-1* strains. (**A**) Northern analysis of *proK* and *proL* using total RNA (15 μg/lane) and probed with probes a (Figure 1A) and c (Figure 2A), respectively. The deduced structures of the processing intermediates are shown to the right of the blot. (**B**) Determination of 5′ and 3′ termini of *proK* and *proL* tRNAs using RT-PCR cloning of 5′-3′ self-ligated transcripts. Each downward arrow represents either a 5′or 3′ end as determined by the sequence analysis. Each upward arrow represents a 3′ end with untemplated poly(A) tail(s). (*) Sequences that have retained their immature 5′ termini.

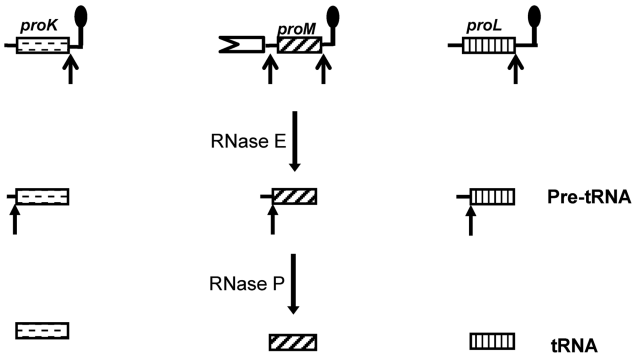

Figure 7. Proline tRNA processing pathways in *Escherichia coli*. RNase E (vertical open arrows) initiate the processing of the primary tRNA transcripts by removing the Rho-independent transcription terminator immediately downstream of the encoded CCA determinant to generate the mature 3′ terminus. In the case of *proM*, RNase E also cleaves at positions 3, 4 and 15 nt upstream of the mature 5′ end to separate the pre-tRNA from the *argX* operon. Endonucleolytic cleavage by either RNase G or RNase Z can weakly substitute for RNase E in the processing of the *proK* and *proM* transcripts. Although endonucleolytic cleavage by RNase P can weakly substitute for RNase E in the processing of *proL*, the 3′ → 5′ exoribonuclease PNPase is very effective in the absence of RNase E. The pre-tRNAs are matured at their 5′ ends by RNase P (vertical closed arrows) to generate the mature tRNAs.

DISCUSSION

Here, we show for the first time that the three primary proline tRNA transcripts in *E. coli* are matured at their 3′ termini without utilizing any of the six 3′ → 5′ exonucleases (RNase T, RNase PH, RNase BN, RNase D, RNase II and PNPase), if RNase E is functionally active. Rather, the proline (*proK*, *proL* and *proM*) primary transcripts are endonucleolytically processed by RNase E immediately downstream of the encoded CCA determinant to generate the mature 3′ termini (Figure 7). This was a surprising result, since RNase E has been previously shown to process many other tRNAs 1–3 nt downstream of the CCA determinant thereby requiring the assistance of one or more of the above exoribonucleases for tRNA 3′-end maturation (8,9,12,44). Subsequently, RNase P cleavages produce the mature 5′ termini resulting in species that can be aminoacylated (Figure 7). By employing a maturation process that does not require any 3′ → 5′ exoribonuclease activity, the proline tRNAs are resistant to polyadenylation by PAP I under most conditions (19,20).

Of particular interest is that RNase E appears to be primarily responsible for the maturation of the *proK* and *proM* 3′ termini, but can be inefficiently replaced by RNase G and RNase Z (Figure 1, Supplementary Figure S3). In contrast, *proL* processing continues in the absence of either all the known endoribonucleases (RNase E, RNase G, RNase Z, RNase LS and RNase P) (Supplementary Figure S3B) or exoribonucleases (RNase T, RNase PH, RNase D, RNase BN/Z, RNase II, PNPase, RNase R) (Figure 2, data not shown). Surprisingly, the *proL* primary transcripts were significantly stabilized in the absence of both RNase E and PNPase with significant reductions in the mature tRNA levels (Figure 4), suggesting that PNPase can effectively process the primary *proL* transcript, particularly in the absence of RNase E.

PNPase has already been shown to be the primary ribonuclease to remove the *leuX* transcription terminator, but it leaves a three nt extension downstream of the CCA terminus that is processed by RNase T to generate the mature 3′ terminus (9). The Rho-independent transcription terminator associated with *leuX* contains four G-C base pairs (bp) out of a 6 bp stem with a net free energy of −9.9 kcal (9), which is considered to be a weak terminator. In contrast, the 12 bp stem associated with *proL* terminator has a net free energy of −15.3 kcal (9). It is thus surprising that not only can PNPase precisely generate the mature *proL* 3′ terminus without the assistance of traditional 3′ → 5′ exoribonucleases, but can also remove the Rho-independent transcription terminator without the participation of the RhlB RNA helicase (data not shown). However, it should be noted that the 12 bp *proL* terminator stem only contains four G-C bp, which is similar to *leuX* (Supplementary Figure S5) and terminators with only four G/C base pairs have been shown to be susceptible to exonucleolytic degradation by both PNPase and RNase II *in vitro* (45). In contrast, both the *proK* (−16.2 kcal) and *proM* (−17.0 kcal) terminator stems contain five or more G-C base pairs (Supplementary Figure S5) with higher free energies (9) and were unaffected by PNPase (Figures 1 and 3, data not shown).

Another major surprise from our findings is that the RNase E cleavages occur immediately downstream of the CCA determinants. In contrast, tRNAs such as *hisR, cysT, pheU, pheV, metT, leuU and metU,* which are also processed by RNase E, retain 1–3 nt downstream of their CCA determinants and become substrates for both 3′ → 5′ exoribonucleases and PAP I (Supplementary Figure S2) (4,19,20). Even the *leuX* monocistronic transcript, which is primarily processed by PNPase at its 3′ end, requires either RNase T or RNase PH for final maturation and is polyadenylated in the absence of these exonucleases (Supplementary Figure S2) (9,19). In fact, the vast majority of tRNA species (79/86) in *E. coli* require the activity of at least RNase T and/or RNase PH for their final 3′ end maturation (19).

These observations raise the interesting question regarding what makes the processing of the three proline tRNAs unique relative to the vast majority of tRNAs in *E. coli*. Extensive analysis of RNase E cleavage sites has suggested a consensus sequence of 5′-(A/G)N↓AU-3′ in which ↓ represents the cleavage site (46–51). As shown in Table 1, all three proline tRNAs have some variation of an RNase E consensus cleavage site, but are cleaved immediately downstream of the CCA determinant. In contrast, *pheU* and *leuU*, both terminating with a Rho-independent transcription terminator, have downstream sequences that are nearly identical to *proK*, but are processed 1–3 nt downstream of the encoded CCA (Supplementary Figure S2 and Table 1). While the downstream sequences of *proM* have no similarity with any of the other tRNAs with a Rho-independent transcription terminator, the downstream sequence of *proL* is similar to *leuX* (Table 1). However, RNase E has no effect on the *leuX* Rho-independent transcription terminator, which has been shown to be processed primarily by PNPase, leaving 3 nt downstream of the CCA determinant (Supplementary Figure S2 and Table 1) (9).

Table 1. 3′ terminal sequences of various tRNAs

tRNA	5′ mature terminus	3′ terminal sequences
proK	C	*G*ACCA↓AAUUCG
pheU	G	*C*ACCAA↓A↓UUCA
leuU	G	*U*ACCAA↓A↓UUCC
proL	C	*G*ACCA↓AAAAUC
leuX	G	*C*ACCAAUUCG[a]
proM	C	*G*ACCA↓AUUUUG

The CCA determinant for each tRNA is underlined. The discriminator nucleotide **A** is in bold. The nucleotide complementary to the 5′ mature sequence is in italics. The downward arrows indicate experimentally identified RNase E cleavage sites.
[a]RNase E does not participate in the processing of the primary *leuX* transcript (9).

Interestingly, direct RNase E cleavage has been demonstrated for mature tmRNA (*ssrA*), which adopts a terminal structure similar to tRNA with a 3′ terminal sequences of CACCA↓A↓A↓AUU in which ↓ represents the RNase E cleavage sites (52). However, the cleavage immediately downstream of tmRNA CCA is considered a minor cleavage site, while the majority of the cleavage takes place 1–2 nt downstream of CCA requiring the assistance of 3′ exonucleolytic trimming (44).

Since the downstream sequences of the *proK* tRNA are almost identical to those of the *pheU* and *leuU* primary transcripts (Table 1), why does RNase E cleave at different locations among the three species? It has previously been noted that most *E. coli* tRNAs have either G, U or A at their mature 5′ termini (19). In contrast, the three proline tRNAs have C at the mature 5′ terminus. As a result the three proline tRNA amino acid acceptor stems would have a GACCA sequence at the 3′ end compared to either CACCA or UACCA for *pheU* and *leuU*, respectively (Table 1). Although it is not clear at this point, it would appear that a 'G' nucleotide upstream of the discriminator base alters the RNase E cleavage specificity downstream of the CCA (Table 1).

It should also be noted that both *proK* and *proL* are transcribed as monocistronic transcripts from multiple transcription initiation sites, which were further upstream than those indicated in the EcoCyc database (21). The primer extension analysis (Supplementary Figure S1) was consistent with the cloning and sequencing data of from over 350 independent cDNA clones obtained from *proK* and *proL* transcripts that had been treated with tobacco acid pyrophosphatase prior to self-ligation where no transcript beyond the initiation sites predicted in Supplementary Figure S1 was obtained (Figures 5 and 6; Supplementary Figure S4). Similar multiple transcription initiation sites have been observed for *secG leuU* transcripts (4) as well as the *pheU* and *pheV* transcripts (Bowden *et al.*, manuscript in preparation).

The Rho-independent transcription terminators associated with all three proline tRNAs appear to be removed first before 5′ end maturation takes place by RNase P. This observation is consistent with the significant accumulation of the full-length transcripts with unprocessed 5′ ends in the presence of wild-type RNase P activity in *rne-1 rph-1, rne-1 Δrng ΔrnZ ΔrnlA rph-1* and *rne-1 rph-1 Δpnp-683* mutants (Figures 1, 2, and 4; Supplementary Figure S3, data not shown). In contrast, inactivation of RNase P in *rnpA49 rph-1* mutant only weakly inhibited removal of the proline tRNAs Rho-independent transcription terminators (Figures 1, 2 and 6B, data not shown).

Finally, although mature proline tRNAs are not, normally, substrates for PAP I (20), under certain conditions unprocessed proline tRNAs can undergo polyadenylation. For example, some *proL* tRNAs in multiple exonuclease mutants have slightly higher molecular weights compared to the wild-type and *rph-1* mutant (Figure 2, compare lanes 3–4 with 1–2). The amount of higher molecular weight *proL* tRNAs was reduced in a corresponding *pcnB* deletion strain (data not shown). Consistent with this result, cloning and sequencing data showed that the 3′ ends of *proL* tRNAs have extended A residues in both the pentuple and sextuple mutants (Figure 5B and Supplementary Figure S4B) compared to the *rph-1* mutant (Figure 5B). While some of these may be due to RNase E cleavage after the encoded A residues few nucleotides downstream of the CCA, others may have been added post-transcriptionally by PAP I. Generally, the percentage of proline tRNAs with mature 5′ ends and 3′ immature ends with templated or untemplated poly(A) tails were low. In contrast, full-length *proK* tRNAs and *proL* tRNAs with immature 5′ ends were subject to increased polyadenylation if these tRNAs could no longer be processed. (Figures 5A and 6B). These results may simply be due to the fact that as the unprocessed tRNAs are not likely to be substrates for aminoacylation, they are increasingly available for polyadenylation by PAP I and most likely enter a quality control pathway (2,19,53).

Taken together, the data presented here show that proline tRNAs are matured at the 3′ end primarily by a direct RNase E endonucleolytic cleavage; thus they do not require the use of any 3′ → 5′ exonuclease, thereby avoiding 3′ polyadenylation. It is possible that other tRNAs, such as the methionines encoded by *metV, metW, metY* and *metZ,* are also matured in a similar way since there is no effect of RNase PH, RNase T and PAP I in their maturation (19).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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