Exoribonuclease and Endoribonuclease Activities of RNase BN/RNase Z Both Function In Vivo*

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Background: In contrast to other RNase Z endoribonucleases, RNase BN is also an exoribonuclease in vitro.

Results: Cells dependent on RNase BN for growth are affected by a single amino acid change that eliminates exoribonuclease activity.

Conclusion: In addition to its endoribonuclease activity, the exoribonuclease of RNase BN also can participate in tRNA maturation.

Significance: RNase BN can serve as a dual-function nuclease in vivo.

SUMMARY

Escherichia coli RNase BN, a member of the RNase Z family of endoribonucleases, differs from other family members in that it also can act as an exoribonuclease in vitro. Here, we examine whether this activity of RNase BN also functions in vivo. Comparison of the X-ray structure of RNase BN with that of Bacillus subtilis RNase Z, which lacks exoribonuclease activity, revealed that RNase BN has a narrower and more rigid channel downstream of the catalytic site. We hypothesized that this difference in the putative RNA exit channel might be responsible for the acquisition of exoribonuclease activity by RNase BN. Accordingly, we generated several mutant RNase BN proteins in which residues within a loop in this channel were converted to the corresponding residues present in B. subtilis RNase Z, thus widening the channel and increasing its flexibility. The resulting mutant RNase BN proteins had reduced or were essentially devoid of exoribonuclease activity in vitro. Substitution of one mutant rbn gene (P142G) for wild type rbn in the E. coli chromosome revealed that the exoribonuclease activity of RNase BN is not required for maturation of phage T4 tRNA precursors, a known specific function of this RNase. On the other hand, removal of the exoribonuclease activity of RNase BN in a cell lacking other processing RNases leads to slower growth and affects maturation of multiple tRNA precursors. These findings help explain how RNase BN can act as both an exo- and an endoribonuclease and also demonstrate that its exoribonuclease activity is capable of functioning in vivo, thus widening the potential role of this enzyme in E. coli.

The biosynthesis of a mature, functional tRNA requires a series of processing steps in which both 5’-leader and 3’-trailer sequences are removed. The 5’-leader sequence is removed by the universal endoribonuclease, RNase P. However, processing of the 3’-trailer sequence differs among organisms (1-9). In those organisms
in which tRNA precursors are devoid of a 3'-CCA sequence, such as eukaryotes, a single endonucleolytic cleavage by RNase Z at the discriminator nucleotide generates a substrate for CCA addition (5, 9). In contrast, in those organisms, such as *Escherichia coli*, in which the CCA sequence is already present in the tRNA precursors, exonucleolytic trimming generates the mature tRNA (3, 10). Thus, the function of RNase Z would appear to be unnecessary in *E. coli*. Surprisingly, however, an RNase Z homologue, termed RNase BN, is present (11-13). As a consequence, it is of considerable interest to understand what the function of RNase BN might be.

RNase BN was originally found to be required for the maturation of those T4 tRNA precursors which lacked a CCA sequence at their 3'-terminus (14). Subsequent studies showed that it is also able to mature CCA-encoded *E. coli* tRNA precursors when all other processing exoribonucleases are absent, although it is very inefficient in this regard (3, 10, 15). Moreover, deletion of the gene encoding RNase BN (*rbn*) in *E. coli* has no effect on cell growth, and it has even been reported that cells lacking RNase BN do not show any alteration in their transcriptome or proteome profile (16), although this remains controversial (17). Nevertheless, under normal circumstances, RNase BN is unlikely to be involved in tRNA maturation *in vivo* except in bacteriophage T4-infected cells (13).

RNase BN was initially reported to be an exoribonuclease based on its ability to remove a mononucleotide residue from the 3' terminus of certain synthetic tRNA precursors (13), in contrast to other members of the RNase Z family which act as endoribonucleases. However, in a recent study, we showed that RNase BN is both a distributive exoribonuclease and an endoribonuclease on model RNA substrates (18). It is strongly inhibited by the presence of a CCA sequence or a phosphoryl group at the 3'-end of RNA (18). The mode of action of RNase BN on tRNA precursors differs based on whether or not the universal 3'-terminal CCA sequence is present. It acts as an endoribonuclease on tRNA precursors that lack a CCA sequence, cleaving after the discriminator nucleotide. In contrast, maturation of tRNA precursors containing a CCA sequence is dependent on the metal ion present; exoribonuclease activity is favored in the presence of Co^{2+}, whereas endoribonuclease activity is stimulated by Mg^{2+} (19). RNase BN cleaves after the CCA sequence in its endoribonucleolytic mode, and it trims off extra 3' residues up to the CCA sequence when acting as an exoribonuclease. In neither case does it remove the CCA sequence from tRNA (19). Interestingly, no other member of the RNase Z family has been reported to have an exoribonuclease activity in addition to the known endoribonuclease activity. However, the mechanism responsible for the dual activities of RNase BN has not been elucidated. Most importantly, it is not known whether the exoribonuclease activity actually functions *in vivo* or whether it is simply an *in vitro* manifestation.

In this study, we identified a structural feature in RNase BN which differs from its RNase Z homologue in *B. subtilis*. Based on this analysis, we were able to generate a mutant form of RNase BN that lacks almost all exoribonuclease activity, but retains its endoribonuclease activity. Expression of this mutant protein in place of wild type RNase BN in *E. coli* had no obvious effect on wild type cells. However, in cells lacking certain other exoribonucleases, and dependent on RNase BN for growth, the presence of the mutant form of RNase BN resulted in even slower growth and affected processing of tRNA precursors. On the other hand, maturation of phage T4 tRNA precursors was unaffected. Biochemical analysis revealed that maturation of the phage T4 tRNA precursors depended solely on the endonucleolytic activity of RNase BN, whereas maturation of host tRNA precursors utilized both activities. Moreover, we found that the endoribonuclease activity of RNase BN cleaved tRNA precursors after the CCA sequence, confirming earlier *in vitro* findings (19). These data indicate that both the exo- and endoribonuclease activities of RNase BN can function *in vivo* and thereby expand the possible roles for this enzyme in *E. coli*.

**EXPERIMENTAL PROCEDURES**

**Materials**—T4 RNA ligase, calf intestine alkaline phosphatase, Nucway™ spin columns and MEGAshortscript™ kit were purchased from
Ambion Inc. T4 polynucleotide kinase, DNase I, and RNase A were obtained from New England Biolabs. ExpressHyb hybridization solution was purchased from Clontech. DpnI was from Fermentas. $[^\gamma-^{32}P]ATP$, $[5'-^{32}P]pCp$ and GeneScreen Plus hybridization transfer membrane were obtained from PerkinElmer Life Sciences. DpnI was from Fermentas. $[^\gamma-32P]ATP$, $[5'-32P]pCp$ and GeneScreen Plus hybridization transfer membrane were obtained from PerkinElmer Life Sciences. The GeneElute™ PCR clean-up kit and bis(p-nitrophenyl) phosphate were from Sigma. The KOD Hot Start DNA polymerase was purchased from Novagen. Sequagel for denaturing urea-polyacrylamide gels was obtained from National Diagnostics. The His-Trap HP column was obtained from GE Healthcare. All other chemicals were reagent grade.

Site-directed Mutagenesis, Overexpression and Purification of the Mutant Protein—Cloning of the wild type \( rbn \) gene into the pET15b plasmid and its overexpression and purification were described previously (12). The mutation P142G was introduced into a plasmid-encoded \( rbn \) gene by site-directed mutagenesis using primers D1 and D2 (Table S1). KOD hot start DNA polymerase was used in the PCR reaction. A template plasmid containing the wild type \( rbn \) gene was then digested by DpnI treatment at 37°C for 2 h. Mutant \( rbn \)-containing plasmids were purified by gel extraction using a gel extraction kit (Qiagen) and transformed into \( E. coli \) BL21(DE3)I/II-. Mutations of P142Q and L143V were also introduced into a plasmid-encoded \( rbn \) gene in the same manner using primers P1 and P2 (for P142Q) and R1 and R2 (for L143V) (Table S1). Mutant proteins were overexpressed in \( E. coli \) strain BL21(DE3)I/II+/pLys and purified using the same procedure used previously for purification of wild type His-tagged RNase BN (12, 18). The purity of wild type and mutant RNase BN proteins was determined on an overloaded SDS-polyacrylamide gel (~3.0 μg of the purified protein). For all of the proteins, a single band at ~35 kDa was observed without any detectable minor contaminating bands.

Bacterial and Phage Strains and Growth Conditions—Wild type \( E. coli \) K-12 strain MG1655(Seq)pH was obtained from laboratory stock and its derivative MG1655(Seq)pHmutS was a gift from Dr. Richard Myers, University of Miami. Deletions of the genes encoding RNases I, II, D or T were introduced into strain MG1655, which is RNase PH-, by transduction with phage P1vir. Mutagenesis of proline142 to glycine of RNase BN in strain MG1655(Seq)pH mutS was performed by recombineering using oligo B1 (Table S1) (20). Recombinants were selected by PCR using primers T1 and T2 (Table S1). A kanamycin resistance gene was also introduced by recombineering after the terminator sequence of the chromosomal \( rbn \) gene (20). The mutant \( rbn \) gene along with the adjacent kanamycin resistance marker from strain MG1655(seq)pHmutS was introduced into wild type MG1655(seq)pH or into MG1655 I/II-D-PH-. Each of these strains was then transformed with the temperature sensitive plasmid pCP20. The kanamycin resistance gene was then flipped out by activation of the flippase encoded in plasmid, pCP20. The P142G mutation in each strain was confirmed by DNA sequencing. Wild type phage T4 and its mutant derivative BU33 were obtained from laboratory stock. BU33 contains an amber mutation in its head protein and a suppressor function for tRNASer as described (14, 21-23). A cca mutation containing the chloramphenicol resistance marker was introduced into strains MG1655 I/II-D and MG1655 I/II-D P142G by transduction with phage P1vir. The rnt mutant gene was subsequently introduced into these strains by P1 transduction.

Cells were grown in YT medium at 37°C. Antibiotics were at the following concentrations: kanamycin, 50 μg/ml; ampicillin, 100 μg/ml; chloramphenicol, 34 μg/ml.

Synthesis and 3'-End Labeling of tRNA Precursors—\( E. coli \) and phage T4 tRNA precursors were synthesized from DNA templates of tRNA genes in \textit{in vitro} transcription reactions using the MEGAscript™ transcription kit as described previously (19). All templates were synthesized from genomic DNAs by PCR with the forward primer containing the T7 RNA polymerase promoter sequence. PCR products were purified using GeneElute™ PCR clean-up kit. Precursor tRNAs were purified by phenol/chloroform/isoamylalcohol (25:24:1) extraction followed by ethanol precipitation as described.
[5'-32P]pCp and T4 RNA ligase were used to label the 3'-end of tRNA precursors in the presence of unlabeled ATP at 4°C for 16 h as described previously. Unincorporated [5'-32P]pCp was removed using a Nucway™ spin column and calf intestine alkaline phosphatase was used to remove the 3'-terminal phosphate from 3'-[32P]pCp-labeled tRNAs. Upon dephosphorylation, 3'-end labeled tRNAs were purified as described (19).

**RNase BN Assay**—3'-[32P]pC-labeled tRNAs (~0.05 μM) or 5'-[32P]-labeled model RNA substrates (10 μM) and 0.14 μM purified wild type or mutant RNase BN, except as otherwise stated in the figure legends, were incubated at 37°C in a 30-μl reaction mixture that contained either 20 mM HEPES, pH 6.5, 200 mM potassium acetate and 0.2 mM CoCl2 or 10 mM Tris-HCl, pH 7.5, 200 mM potassium acetate and 5 mM MgCl2 (19). Portions were taken at the indicated times, and the reaction was terminated by the addition of 2 volumes of gel loading buffer (90% formamide, 20 mM EDTA, 0.05% SDS, 0.025% bromophenol blue and 0.025% xylene cyanol). Reaction products were resolved on 20% denaturing 7.5 M urea polyacrylamide gels and visualized using a STORM 840 phosphorimaging device (GE Healthcare). ImageQuant (GE Healthcare) was used to quantitate the bands.

**Phosphodiesterase Assay**—Bis(p-nitrophenyl) phosphate was used as substrate to determine the phosphodiesterase activity of RNase BN. Standard reaction conditions were 20 mM Tris-HCl (pH 7.4), 2 mM substrate, 2.0 μg of purified His-tagged wild type or mutant RNase BN, and 5 mM MgCl2. Release of p-nitrophenol (ε=11500 M⁻¹ cm⁻¹ at pH 7.4) was continuously monitored for 3 min at 405 nm. One unit of activity corresponds to 1 μmol of p-nitrophenol liberated per minute at 37°C.

**Phage Assay**—Fifty microliters of overnight culture of wild type or mutant E. coli cells were incubated with 10 μl of either bacteriophage T4 or T4 mutant strain BU33 (10⁸ plaque-forming unit/μl) at 37°C for 5 min. The suspension was then overlaid onto LB plates with 2.5 ml of top agar. Plates were incubated overnight at 37°C prior to counting plaques.

**RNA preparation and Northern Blotting**—Cells were grown in YT medium to an A600 ~ 1.0. Total cellular RNA was isolated by phenol extraction as described (24). RNA samples from wild type and mutant strains, containing the same amount of RNA, were dissolved in gel loading buffer and loaded on a 6% denaturing 7.5 M urea-polyacrylamide sequencing gel. The gel was run at 1000 V until the xylene cyanol dye had migrated ~30 cm. The RNA was transferred to a GeneScreen plus membrane by horizontal transfer for 1.5 h at 150 mA using 0.5X Tris borate/EDTA as the transfer solution. T4 polynucleotide kinase was used to prepare a 5'-32P-labeled DNA oligonucleotide probe complementary to the 5'-end of the tRNA. Hybridization of the probe to the transferred RNA was carried out by overnight incubation in ExpressHyb hybridization solution and the detected bands were visualized by phosphorImager analysis (GE Healthcare).

**RESULTS**

In earlier work (18, 19), we showed that RNase BN can mature E. coli tRNA precursors in vitro using either an exo- or endoribonucleolytic mode of action. However, it has been unclear how the enzyme carries out this dual mode of action. It is also not known whether both activities actually function in vivo. Since, the ability of RNase BN to function as both an exo- and an endoribonuclease differs from other members of the RNase Z family, we reasoned that comparison of RNase BN with another RNase Z member might shed light on the explanation for their different catalytic properties.

**Structural Comparison of RNase BN and an RNase Z**—The crystal structures of E. coli RNase BN and its B. subtilis homologue (RNase Z) have been solved (25-27). Each enzyme is a dimer containing a core Zn-dependent β-lactamase domain with a HXHDXH metal binding motif, as well as additional His and Asp residues that contribute coordination sites to the metal ions. Each of the subunits possesses a protruding flexible arm which is believed to have a role in tRNA binding (25-28). Both subunits of RNase BN from E. coli appear to be identical, whereas those of B. subtilis RNase Z maintain a different
conformation despite having identical sequences (25, 27).

To identify structural differences between the E. coli and B. subtilis proteins that might account for their catalytic differences, we superimposed their crystal structures as shown in Fig. 1. The region encompassing the two metal binding sites, the putative site of catalysis, also contains an RNA-binding channel (9, 26). RNA bound in this channel is presumed to be cleaved at the site of metal binding. The cleavage product would then diffuse away through the putative exit channel, which is the portion of the RNA-binding channel that lies downstream of the catalytic site and through which the cleavage product will leave the enzyme. For endonucleolytic cleavage, we propose that the RNA molecule would lie in the RNA binding channel in a manner such that its 3' end extends well into the exit channel. As a consequence, the catalytic site would access and cleave an internal phosphodiester bond. On the other hand, if the 3' end of the RNA cannot extend beyond the catalytic site, exonucleolytic cleavage of the terminal 3' nucleotide would be favored. Thus, we suggest that the position of the RNA substrate, determined by the extent of its entry into the putative exit channel, would result in either endonucleolytic or exonucleolytic action on the RNA chain. Structural features which impede or promote RNA access into the exit channel would thereby favor one or the other catalytic activity.

Comparison of the putative exit channel regions of E. coli RNase BN and B. subtilis RNase Z (9) indicates that in the E. coli protein, a loop extending into the exit channel (shown in yellow in Fig. 1) just downstream of the catalytic site narrows the exit channel more than the corresponding loop in the B. subtilis protein (red). This loop in E. coli RNase BN also contains a proline (P142) which should make it much more rigid than the B. subtilis loop which has a glycine at the corresponding position. Other regions around the catalytic site of the E. coli and B. subtilis proteins are otherwise very similar. Thus, we hypothesized that the loop and especially proline 142, which could impede an RNA substrate from entering into the exit channel, might be responsible for promoting exonucleolytic activity in E. coli RNase BN.

Isolation and Activity of RNase BN Mutant Proteins– To examine the importance of the putative exit channel loop and its flexibility on the activity of RNase BN, we generated several mutations within the loop and purified the resulting proteins. These included conversion of Pro142 to either glycine or glutamine, and a change of Leu143 to Val. The mutations of Pro142 would be expected to increase the flexibility of the loop, and to also substitute this residue with either the small glycine residue present in the B. subtilis protein or with a relatively large residue, glutamine. The Leu143 to Val change was expected to slightly widen the exit channel and to convert this residue to that present in the B. subtilis enzyme. As an initial screen, the mutant proteins were compared to wild type RNase BN for its three known activities, i.e., endoribonuclease, exoribonuclease, and phosphodiesterase (Table 1) using tRNASelC precursor as substrate for the nuclease activities and bis-\(p\)-nitrophenyl phosphate as substrate for the diesterase activity.

The data presented in Table 1 show that all of the loop mutants retain, or even increase, their endoribonuclease activity. In contrast, exoribonuclease activity is essentially eliminated in the two P142 mutant proteins, and is reduced about 3-fold in the L143V mutant protein. Interestingly, even though exoribonuclease activity is dramatically reduced, phosphodiesterase activity is unaffected in all the mutants. These initial assays support a model in which mutation of P142 increases the flexibility of the loop thereby effectively widening the exit channel and enabling the 3' end of the tRNA precursor substrate to bind mainly within the exit channel which promotes the endo activity and inhibits exo activity. A similar effect was seen with the LI43V mutant protein, which also would be expected to widen the exit channel, but the effect is less pronounced. Since the small substrate used in the phosphodiesterase assay is likely confined to the vicinity of the catalytic site, this activity is unaffected by mutations in the putative exit channel. As both Pro142 mutant proteins were essentially the same with regard to loss of exoribonuclease activity, all subsequent studies were carried out with the P142G mutation.

Effect of P142G Mutation in RNase BN on its Catalytic Activity– To further analyze the apparent
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loS of exoribonuclease activity in the P142G mutant protein, it was compared to the wild type enzyme using a variety of known RNase BN substrates. We first tested two synthetic RNA substrates, A_{17} and G_{5}A_{12}CCA-\text{A}_{5}, previously shown to be acted on by RNase BN in an exonucleolytic manner (18). However, as shown in Fig. 2, these substrates were resistant to the P142G mutant protein, whereas they were active substrates of the wild type enzyme, confirming that the P142G mutant enzyme is essentially devoid of exoribonuclease activity.

The wild type and mutant RNase BN proteins were further analyzed using 3'-\[^{32}\text{P}\]pC labeled tRNA^{\text{SelC}} and tRNA^{\text{PheV}} precursors from E. coli as substrates (prepared as described in “Experimental Procedures”). With pre-tRNA^{\text{SelC}} as substrate (containing 5 nt following the CCA sequence), wild type RNase BN generates approximately equal amounts of exo- and endoribonucleolytic cleavage products in the presence of Mg^{2+}. In contrast, essentially only the endonucleolytic cleavage product is produced by mutant RNase BN under the same conditions (Fig. 3A). In the presence of Co^{2+}, which strongly favors the exoribonucleolytic activity of RNase BN (18), both the wild type and mutant enzymes were able to remove the 3’ terminal mononucleotide from the tRNA^{\text{SelC}} precursor (Fig. 3A). However, even in this sensitive assay, the mutant RNase BN exoribonuclease activity was reduced ~20-fold compared to the wild type enzyme.

The second E. coli tRNA precursor examined, tRNA^{\text{PheV}}, which contains 7 extra residues following the CCA sequence, is matured primarily by the endoribonucleolytic activity of RNase BN (19). As shown in the Fig. 3B, mutant RNase BN also acts primarily endoribonucleolytically on this substrate in the presence of Mg^{2+}. However, when the assay is carried out in the presence of Co^{2+}, major differences between wild type and mutant enzymes are observed (Fig. 3B). Whereas the wild type protein now produces essentially only the exoribonuclease-generated CMP product, mutant RNase BN continues to act primarily as an endoribonuclease with only a very low level of exoribonuclease activity, even under conditions which strongly promote this mode of action. Thus, for all substrates tested, and even in the presence of Co^{2+}, conversion of P142 to G in RNase BN strongly inhibits its exoribonuclease activity. Based on these observations, we anticipate that the P142G mutant RNase BN would function as an endoribonuclease on tRNA precursors in vivo.

Construction of Strains Lacking the Exoribonuclease Activity of RNase BN—Although RNase BN displays both exo- and endoribonucleolytic activities in vitro, it is not known whether both activities can function in vivo. The availability of a mutant RNase BN that lacks the exoribonuclease activity enabled us to address this question. Accordingly, the P142G mutation was first introduced into strain MG1655(seq)^{rph+} by recombineering (20). The mutant rbn gene was subsequently transferred into a strain lacking RNases I, II, D, T and PH. To ensure that the mutant gene was expressed normally, we compared the level of phosphodiesterase activity in the rbn^{+} and P142G derivatives of the multiple RNase-deficient strain. Since the phosphodiesterase activity is unaffected by the P142G mutation, the amount of this activity is a direct measure of the amount of RNase BN protein present in each strain.

As can be seen in Table S2, there is no significant difference in the level of phosphodiesterase activity between extracts of the wild type and the P142G mutant strain. Moreover, based on the small amount of phosphodiesterase activity remaining in a rbn null strain, ~90% of the activity in the extract is due to RNase BN. Thus, based on these data, we conclude that P142G RNase BN is expressed normally and is active.

Effect of P142 to G Mutation on the Maturation of Phage T4 tRNA Precursors in vivo—One known function of RNase BN is the maturation of those phage T4 tRNA precursors that lack a 3’-CCA sequence (14, 22). Thus, an E. coli strain deficient in RNase BN is unable to support growth of mutant T4 phage strain BU33 due to its inability to process the 3’-end of a required phage-encoded suppressor tRNA^{\text{Ser}} (14). However, it is not known whether the exo- or endoribonucleolytic activity of RNase BN is important for this action on phage T4 tRNA precursors in vivo. To examine this question, wild type phage T4 and T4 phage BU33
were each plated on wild type \textit{E. coli}, on a RNase BN deficient strain or on the strain that is deficient in the exo activity of RNase BN. The data presented in the Table 2 show that wild type phage T4 is able to form plaques on all three strains with equal efficiency. Likewise, T4 phage BU33 generates nearly the same number of plaques on wild type and on the P142G mutant strain. In contrast, a strain with a deletion of the \textit{rbn} gene cannot support BU33 growth. These data show that elimination of the exoribonuclease activity of RNase BN does not affect the plating efficiency of either wild type T4 phage or phage BU33 suggesting that the exoribonuclease activity of RNase BN is not required for the maturation of phage T4 tRNA\textsubscript{Ser}.

To obtain further support for this conclusion, we prepared by \textit{in vitro} transcription the T4 phage dimeric tRNA\textsubscript{Pro-Ser} precursor (Fig. 4), known to be a substrate for 3'-processing by RNase BN \textit{in vivo} (14, 22, 23). The substrate was labeled by ligation of [\textsuperscript{32}P]pCp to its 3' terminus, followed by removal of the 3'-phosphoryl group by phosphatase. The resulting labeled precursor was then treated with either purified wild type RNase BN or the P142G exoribonuclease-deficient mutant enzyme, and the products were analyzed on a 20\% denaturing acrylamide gel. The major product produced with both enzymes is a tetranucleotide, with no [\textsuperscript{32}P]CMP evident (Fig. 4). These data confirm that RNase BN removes the extra 3' terminal nucleotides from the dimeric precursor in an endoribonucleolytic manner. Note also that endoribonuclease activity is elevated in the mutant enzyme, amounting to ~5-fold with this substrate.

\textbf{Effect of P142 to G Mutation on the Maturation of \textit{E. coli} tRNA Precursors in vivo}– It is unlikely that RNase BN is involved in the maturation of \textit{E. coli} tRNA precursors under normal conditions because it is less efficient in removing extra residues from the 3'-end of pre-tRNAs than other tRNA processing enzymes present in the cell. However, in the absence of the other tRNA 3'-processing enzymes, tRNA maturation becomes dependent on RNase BN (15), providing a system to determine the effect of removing its exoribonuclease activity. Thus, in a genetic background in which RNases I, II, D, T and PH are absent, we performed Northern blot analysis on multiple tRNAs in strains containing or lacking the exoribonuclease activity of RNase BN.

Fig. 5 shows the results obtained with one of these tRNAs, tRNA\textsubscript{PheV}. tRNA precursors do not accumulate to a detectable level in the wild type strain in which all the tRNA processing RNases are present. The amount of tRNA precursors increases to ~5% when the exoribonucleases, RNase II, D, T and PH are removed. Additional removal of the exoribonuclease activity of RNase BN leads to increased accumulation of the PheV precursor, amounting to ~30% of the total tRNA\textsubscript{PheV} species present. These observations indicate that the exoribonuclease activity of RNase BN participates in maturation of the tRNA\textsubscript{PheV} precursor \textit{in vivo}.

A similar analysis was carried out for 9 additional tRNAs (Table 3). As can be seen, 5 of these 9 tRNAs display a significant increase in the amount of tRNA precursor present when RNase BN lacks its exoribonuclease activity. These data show that the exoribonuclease activity of RNase BN can function \textit{in vivo} and can contribute to the maturation of tRNA precursors. However, since mature tRNA is present for all the tRNAs studied, it is likely that the intact endoribonuclease activity of RNase BN provides a significant portion of overall tRNA processing activity under these special conditions. Moreover, these data also indicate that the relative importance of the endo and exo activities of RNase BN varies depending on the identity of the tRNA precursor examined. The reasons for these differences among precursors are not understood, but it is well known that tRNA precursors respond very differently to removal of individual processing exoribonucleases (3).

\textbf{Effect of the P142 to G Mutation in RNase BN on Cell Growth}– Deletion of the gene encoding RNase BN (\textit{rbn} gene) has no effect on growth of \textit{E. coli}. However, its presence is required in a cell lacking other tRNA 3'-end processing exoribonucleases (II, D, T, PH) since additional deletion of the \textit{rbn} gene makes the cell inviable. Although the presence of RNase BN maintains viability, cells grow poorly, with a doubling time of 75-120 min depending on the genetic
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RNase BN Cleaves tRNA Precursors after the CCA Sequence in vivo– As noted above, the endo activity of RNase BN processes tRNA precursors in vivo, but its site of cleavage was not known. RNase BN does not remove the CCA sequence from either precursor or mature tRNAs in vitro (18), and it was of interest to determine whether the same mode of action is operative in vivo. To answer this question, we introduced a cca mutation by P1 transduction eliminating the enzyme, tRNA nucleotidyltransferase. We hypothesized that if RNase BN cleaves tRNA precursors after the discriminator nucleotide, tRNA nucleotidyltransferase would be required to add back the CCA sequence to generate a mature tRNA, whereas this enzyme would not be needed if the cleavage were after the CCA sequence. We found that introduction of a cca mutation into cells containing wild type RNase BN or containing the P142G mutant RNase BN and lacking other 3’-processing exoribonucleases, has no effect on the growth rate (data not shown), strongly suggesting that cleavage is after the CCA sequence in each case. To confirm these data, Northern blot analysis was carried out on a series of tRNAs. As can be seen, removal of processing nucleases leads to accumulation of tRNA precursors. However, none of the tRNAs tested show any shorter species devoid of the CCA sequence in the tRNA nucleotidyltransferase mutants (Fig. 7). Moreover, removal of tRNA nucleotidyltransferase from the RNase-deficient strain has no effect on the amount of mature tRNA generated. These results indicate that RNase BN does not remove the CCA sequence from tRNA in vivo, and therefore, must cut after the CCA sequence.

DISCUSSION

RNase BN, unlike other RNase Z homologues, has dual endo- and exoribonuclease activities (18). In this study, we examined the role of each of these activities in vivo. We showed that (a) conversion of a single proline residue to glycine or glutamine in the putative exit channel essentially eliminates the exoribonuclease activity of RNase BN in vitro; (b) the exoribonuclease activity of RNase BN is not required for maturation of phage T4 tRNA precursors in vivo; (c) the absence of RNase BN exoribonuclease activity results in slower growth of a cell which lacks other processing exoribonucleases; (d) the exoribonuclease activity of RNase BN participates in E. coli tRNA processing; and (e) the endoribonuclease activity of RNase BN cleaves after the CCA sequence in E. coli tRNA precursors.

Based on the known crystal structures of E. coli RNase BN (27), which displays both endo- and exoribonuclease activities and of B. subtilis RNase Z (25), which is only an endoribonuclease (5), we suspected that the observed differences in the width and flexibility of their putative exit channels might play a role in their different catalytic properties. Accordingly, we individually
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mutated two residues within a loop protruding into this channel of RNase BN. As predicted, the resulting mutant RNase BN proteins had reduced or essentially no exoribonuclease activity, although endoribonuclease activity remained intact, or even increased slightly. These findings provide important clues about the structural basis for the distinct catalytic properties of exo- and endoribonucleases, and warrant more detailed investigation. However, for the purpose of the present study, the generation of a mutant RNase BN, essentially devoid of exoribonuclease activity, enabled us to examine the functional role of each of RNase BN’s activities in vivo.

Elimination of the exoribonuclease activity of RNase BN did not affect the growth of a wild type strain. Undoubtedly, the exoribonucleases present are able to take over any exo function of RNase BN. However, in a strain that lacks the other 3’ processing exoribonucleases, the absence of the exo activity of RNase BN causes growth to slow even further, and also affects maturation of certain tRNA precursors. This is the only example in which the exoribonuclease activity of an RNase Z homologue has been shown to have a function in vivo. In contrast, the exo activity of RNase BN is not required for maturation of phage T4 tRNA precursors, the major known function of this RNase, even when the other tRNA-processing exoribonucleases are absent. This finding is consistent with our previous in vitro observations that RNase BN acts primarily as an endoribonuclease on tRNA precursors that lack a CCA sequence at their 3’-end (19). In fact, the data presented here indicate that RNase BN can act as an endoribonuclease even on CCA-containing tRNA precursors inasmuch as an RNase-deficient cell is able to survive in the absence of the RNase BN exo activity. Thus, of the tRNA precursors tested, the endoribonuclease activity of RNase BN is able to generate as much as 40-90% of the normal levels of mature tRNAs when the exo activity is absent. Moreover, complete removal of RNase BN in a strain lacking the other processing exoribonucleases leads to inviability indicating that no other tRNA 3’ processing activity is present (15).

Interestingly, while all RNase Z homologues exhibit endoribonuclease activity, their site of action on tRNA precursors may differ. Eukaryotic members of the family all cleave after the discriminator nucleotide (9), as does B. subtilis RNase Z (5). In eukaryotic cells and in those tRNA precursors which are the substrates of RNase Z in B. subtilis, no CCA sequence is present. In contrast, both the Thermotoga maritima and E. coli enzymes cleave after the CCA sequence (7, 19, present results), and in these organisms, all but one (in T. maritima) tRNA precursor contain an encoded CCA sequence. Based on this information it appears that the catalytic properties of the organism’s RNase Z have co-evolved with whether or not the CCA sequence is encoded, as it would be energetically wasteful to first remove, and then re-synthesize, the CCA sequence. In fact, a CCA sequence strongly inhibits both the exo- and endoribonuclease activities of the E. coli enzyme (18, 19). However, the mechanism by which a CCA sequence inhibits catalysis by RNase BN remains to be determined.

Although RNase BN is the only known member of the RNase Z family which acts as both an exo- and an endoribonuclease, two other RNases belonging to the β-lactamase family were reported to have such a dual function (29, 30). These are RNase J from B. subtilis (29) and CPSF 73 (30) from mammalian cells, an RNase that functions in histone mRNA processing. However, in contrast to RNase BN which acts 3’ to 5’, each of these enzymes functions as a 5’-3’ exoribonuclease. Moreover, both RNase J1 and CPSF 73, unlike RNase BN, are essential for cell survival under normal conditions.

The results presented here add considerably to our knowledge of RNase BN and its capabilities and substrate specificity in vivo. However, its primary function in E. coli remains unknown. The fact that it can function both as an exo- and endoribonuclease opens up additional possibilities for its role in vivo.
REFERENCES


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**FOOTNOTES**

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**FIGURE LEGENDS**

**Figure 1**: Loop region around Prol42 in *E. coli* RNase BN obstructs the RNA exit channel. Superimposed structures of *E. coli* RNase BN (PDB ID: 2CBN) and *B. subtilis* RNase Z (PDB ID: 2FK6) are shown in ribbon representations, colored as gray and pale green, respectively. Loop region 140-145 is highlighted in yellow for *E. coli* RNase BN and in red for *B. subtilis* RNase Z. The orientation shown looks into the RNase BN active site, with the putative exit channel on the back top of the figure. Residue P142 in *E. coli* (EC) RNase BN is shown as sticks, and the position of the corresponding glycine in *B.
Activities of RNase BN in vivo

subtilis (BS) RNase Z is marked by a red sphere. The two zinc ions coordinated at the E. coli RNase BN active site are shown as blue spheres.

Figure 2: Comparison of the activity of wild type and P142G mutant RNase BN on model RNA substrates: The reactions were carried out as described under “Experimental Procedures” with the addition of 1.7 μM of purified wild type or mutant RNase BN and 10 μM 5\(^-\)\(^{32}\)P-labeled single stranded A\(_{17}\) or G\(_{5}\)A\(_{12}\)CCA-A\(_{4}\) substrate in the presence of Mg\(^{2+}\). Portions were withdrawn at indicated time points.

Figure 3: Processing of tRNA precursors by wild type and mutant RNase BN. A. Shown is the structure of the E. coli tRNA\(^{Selo}\) precursor with the 3’-terminal CCA sequence in boldface. tRNA\(^{Selo}\) (0.05 μM) labeled with \([^{32}\)P]pC at its 3’-end was treated with wild type or mutant (P142G) RNase BN (0.14 μM) in the presence of either Mg\(^{2+}\) at pH 7.5 or Co\(^{2+}\) at pH 6.5. Cleavage products were analyzed by 20% denaturing PAGE. The positions of the 5-nt endoribonucleolytic cleavage product and the mononucleotide generated as a result of exoribonucleolytic trimming are indicated. B. The structure of the E. coli tRNA\(^{PheV}\) precursor is shown with the 3’-CCA sequence in boldface. 3’-\(^{32}\)P]pC labeled tRNA\(^{PheV}\) was treated with wild type or mutant RNase BN and analyzed as described for panel A. The 7-nt endoribonucleolytic cleavage product and the mononucleotide generated as a result of exoribonucleolytic trimming are indicated. Identification of the reaction products was determined with RNA oligonucleotides and with CMP as standards. The mononucleotide exonuclease product was also confirmed to be CMP by paper chromatography, as described (18, 19).

Figure 4: Processing of phage tRNA\(^{Pro-Ser}\) precursor by RNase BN. The structure of phage T4 tRNA\(^{Pro-Ser}\) dimeric precursor is shown in the figure with the discriminator nucleotides in boldface. Phage T4 tRNA\(^{Pro-Ser}\) precursor (12 nM), labeled at its 3’-end with \([^{32}\)P]pC as described under “Experimental Procedures,” was used as the substrate. Digestion with wild type and P142G mutant RNase BN (0.04 μM) was carried out in the presence of Mg\(^{2+}\) at pH 7.5. All bands shorter than the full-length tRNA\(^{Pro-Ser}\) precursor are incomplete transcripts that are labeled with \([^{32}\)P]pCp.

Figure 5: Northern analysis of tRNA\(^{PheV}\). Total RNA samples were isolated from 10 ml of culture as described in “Experimental Procedures” and dissolved in gel loading buffer. Eight μg of RNA samples from each of strains MG1655 I I D’T-, MG1655 I I D’T containing the P142G mutation in RNase BN and wild type MG1655(seq)\(^{rph}\) were subjected to electrophoresis and Northern analysis as described in “Experimental Procedures”. Hybridization was at 37°C overnight.

Figure 6: Effect of P142G mutation in RNase BN on cell growth in an exoribonuclease deficient strain. Strains MG1655 I I D’T PH and MG1655 I I D’T PH containing the P142G mutation in RNase BN were grown in YT medium at 37°C. Samples were withdrawn at various times to measure cell growth (A\(_{600}\)). The growth rate is plotted as the log of A\(_{600}\) values as a function of time.

Figure 7: Northern blot analysis of tRNA\(^{Cys}\), tRNA\(^{Tyr}\) and tRNA\(^{Ala}\). RNA samples were prepared as described in “Experimental Procedures”. Eight micrograms of total RNA were subjected to electrophoresis and then transferred to a GeneScreen plus membrane. Transfer RNAs were detected using a \(^{32}\)P-labeled 5’-end specific probe.
Activities of RNase BN in vivo

<table>
<thead>
<tr>
<th>Protein</th>
<th>Endoribonuclease Activity (%)</th>
<th>Exoribonuclease Activity (%)</th>
<th>Phosphodiesterase Activity (%)</th>
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<tbody>
<tr>
<td>Wild Type</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>P142G</td>
<td>161 ± 11</td>
<td>5 ± 1</td>
<td>98 ± 4</td>
</tr>
<tr>
<td>P142Q</td>
<td>122 ± 9</td>
<td>9 ± 2</td>
<td>99 ± 3</td>
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<tr>
<td>L143V</td>
<td>102 ± 4</td>
<td>33 ± 9</td>
<td>99 ± 2</td>
</tr>
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</table>
**Table 2**

**Plating of bacteriophage T4 on wild type and mutant *E. coli***- Plaque assays of wild type T4 and T4 mutant BU33 were carried out as described in “Experimental Procedures”. Equal amounts of either T4 or BU33 were used with each *E. coli* strain. A representative experiment from 3 independent measurements is shown.

<table>
<thead>
<tr>
<th><em>E. coli</em> Strain</th>
<th>Relevant Phenotype</th>
<th>T4</th>
<th>BU33</th>
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<tr>
<td>MG1655(seq)<em>rph</em>+</td>
<td>Wild Type</td>
<td>126</td>
<td>141</td>
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<tr>
<td>MG1655(seq)<em>rph</em>+ P142G</td>
<td>RNase BN Exo-mutant</td>
<td>117</td>
<td>122</td>
</tr>
<tr>
<td>MG1655(seq)<em>rph</em>+ rbn*</td>
<td>RNase BN*</td>
<td>108</td>
<td>0</td>
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</table>
Table 3

Accumulation of precursor tRNAs in the P142G exoribonuclease-deficient strain. Quantitation of the Northern analyses was done by ImageQuant (GE Healthcare). Data are expressed as the percentage of total radioactivity present for each tRNA. Values shown are the average of 3 separate experiments.

<table>
<thead>
<tr>
<th>tRNA Species</th>
<th>% Precursor P142G</th>
<th>% Precursor P142G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe V</td>
<td>6.1 ± 1.5</td>
<td>27.5 ± 4.0</td>
</tr>
<tr>
<td>AlaU</td>
<td>18.4±5.0</td>
<td>43.8 ± 8.2</td>
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<tr>
<td>Tyr1</td>
<td>22.0 ± 3.7</td>
<td>42.3 ± 7.0</td>
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<tr>
<td>TyrU</td>
<td>4.9 ± 1.0</td>
<td>26.1 ± 4.3</td>
</tr>
<tr>
<td>ArgX</td>
<td>14.3 ± 2.3</td>
<td>28.5 ± 6.5</td>
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<tr>
<td>CysT</td>
<td>11.8 ± 2.8</td>
<td>26.9 ± 4.1</td>
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<tr>
<td>LeuZ</td>
<td>16.0 ± 3.0</td>
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<tr>
<td>Val2A</td>
<td>42.8 ± 4.2</td>
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<tr>
<td>Ser3</td>
<td>52.1 ± 6.7</td>
<td>46.7 ± 4.6</td>
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<tr>
<td>Sel-Cys</td>
<td>27.3 ± 3.7</td>
<td>23.0 ± 4.4</td>
</tr>
</tbody>
</table>
Figure 1

Activities of RNase BN in vivo

Exit Channel

BS Gly141

EC Pro142
Figure 2

Activities of RNase BN in vivo
Figure 3

Activities of RNase BN in vivo

A. 5'-GCCA AAAU *C-3'

B. 5'-GGGAG A CCA CUAAUU *C-3'

<table>
<thead>
<tr>
<th></th>
<th>Mg²⁺</th>
<th>Co²⁺</th>
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<tr>
<td>WT</td>
<td>P142G</td>
<td>WT</td>
</tr>
<tr>
<td>Time (min)</td>
<td>0 15 30</td>
<td>0 3 6 9 12</td>
</tr>
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</table>

Endo- 5 nt
Exo- CMP
Figure 4

Activities of RNase BN in vivo
Figure 5

Activities of RNase BN in vivo
Figure 6
Activities of RNase BN in vivo

Figure 7

<table>
<thead>
<tr>
<th></th>
<th>tRNA_{Cys}T</th>
<th>tRNA_{Tyr}U</th>
<th>tRNA_{Ala}U</th>
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<tr>
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<td><img src="image14" alt="Image" /></td>
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+2-4 nt