Growth Phase-dependent Variation of RNase BN/Z Affects Small RNAs

REGULATION OF 6S RNA

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RNase BN, the RNase Z family member in E. coli, can participate in the processing of tRNA precursors. However, this function only becomes apparent when other processing enzymes are absent, raising the question of its primary physiological role. Here, we show that RNase BN itself is subject to growth phase-dependent regulation, because both rbn mRNA and RNase BN protein are at their highest levels in early exponential phase, but then decrease dramatically and are essentially absent in stationary phase. As a consequence of this variation, certain small RNAs, such as 6S RNA, remain low in exponential phase cells, and increase greatly in stationary phase. RNase BN affects 6S RNA abundance by decreasing its stability in exponential phase. RNase BN levels increase rapidly as cells exit stationary phase and are primarily responsible for the decrease in 6S RNA that accompanies this process. Purified RNase BN directly cleaves 6S RNA as shown by in vitro assays, and the 6S RNA:pRNA duplex is an even more favorable substrate of RNase BN. The exoribonuclease activity of RNase BN is unnecessary because all its action on 6S RNA is due to endonucleolytic cleavages. These data indicate that RNase BN plays an important role in determining levels of the global transcription regulator, 6S RNA, throughout the growth cycle.

The RNase Z family of enzymes is widespread among organisms from prokaryotes to eukaryotes. These RNases generally participate in the 3’ processing of tRNA precursors lacking an encoded CCA sequence, cleaving right after the discriminator nucleotide to generate a substrate for CCA addition by tRNA nucleotidyltransferase (1). In addition to tRNA precursors, some mRNAs have also been identified as substrates including some in rice (2) and Escherichia coli (3), raising the possibility of these enzymes acting as important regulators of small RNAs (sRNAs) by participating in their maturation or turnover (12, 13). 6S RNA, a stable sRNA, is an important transcription regulator that acts by binding to the sigma 70-containing holoenzyme of RNA polymerase (Eρ70) and reducing its activity (14). In late stationary phase, 6S RNA accumulates to high levels and binds the vast majority of Eρ70, leading to reduced transcription of many ρ70-dependent genes (15). The mechanism driving accumulation of 6S RNA in stationary phase remains unclear. Although regulated transcription contributes to its accumulation in stationary phase (16), increased stability was also suggested to be an important determinant (14). However, ribonucleases that affect 6S RNA stability have not yet been identified in E. coli or any other bacterium (17). Interestingly, 6S RNA is also used as a template by RNA polymerase to produce a small RNA, termed pRNA. The synthesis of pRNA is most apparent during outgrowth, leading to dissociation of a 6S RNA:pRNA duplex from the polymerase (18). The released RNA polymerase functions to increase transcription during outgrowth, whereas the released 6S RNA:pRNA is rapidly degraded by as yet unidentified ribonucleases.

In this study, we find that RNase BN regulates the levels of certain sRNAs, such as 6S RNA, during exponential phase. We first show that RNase BN itself is regulated, being present at a

the CCA trinucleotide (5), obviating a need for the action of RNase Z.

The RNase Z family member in E. coli is termed RNase BN and was originally identified as an enzyme required for maturation of those bacteriophage T4 tRNA precursors that lacked a CCA sequence (6, 7). In contrast to RNase Z in most organisms, RNase BN can act as an exoribonuclease or an endoribonuclease (8, 9). When acting on CCA-containing tRNA precursors, RNase BN cleaves after the CCA sequence using its endoribonuclease activity or trims up to the CCA sequence using its exoribonuclease activity, keeping the CCA sequence intact (10). Although both activities of RNase BN can function in vivo (9), a role for this enzyme in maturation of tRNA precursors only becomes evident when other processing ribonucleases are inactivated (11), suggesting that its primary function in wild type E. coli cells is still unknown.

Ribonucleases play an important role in cellular RNA metabolism, and recent studies have revealed that these enzymes may act as important regulators of small RNAs (sRNAs) by participating in their maturation or turnover (12, 13). 6S RNA, a stable sRNA, is an important transcription regulator that acts by binding to the sigma 70-containing holoenzyme of RNA polymerase (Eρ70) and reducing its activity (14). In late stationary phase, 6S RNA accumulates to high levels and binds the vast majority of Eρ70, leading to reduced transcription of many ρ70-dependent genes (15). The mechanism driving accumulation of 6S RNA in stationary phase remains unclear. Although regulated transcription contributes to its accumulation in stationary phase (16), increased stability was also suggested to be an important determinant (14). However, ribonucleases that affect 6S RNA stability have not yet been identified in E. coli or any other bacterium (17). Interestingly, 6S RNA is also used as a template by RNA polymerase to produce a small RNA, termed pRNA. The synthesis of pRNA is most apparent during outgrowth, leading to dissociation of a 6S RNA:pRNA duplex from the polymerase (18). The released RNA polymerase functions to increase transcription during outgrowth, whereas the released 6S RNA:pRNA is rapidly degraded by as yet unidentified ribonucleases.

In this study, we find that RNase BN regulates the levels of certain sRNAs, such as 6S RNA, during exponential phase. We first show that RNase BN itself is regulated, being present at a
Regulation of 6S RNA by RNase BN

Results

Variation in rbn mRNA and RNase BN Protein with Growth—As a first step to understand the physiological function of RNase BN in *E. coli*, we analyzed its chromosomal expression during different phases of growth. Samples were taken from early exponential phase to late stationary phase for measurement of *rbn* message and FLAG-tagged RNase BN protein. *rbn* mRNA, as detected by RT-PCR (Fig. 1A), was found to be at its highest level from early to mid-exponential phase (\(A_{600} = 0.1–0.5\)). After that time, *rbn* mRNA decreased rapidly to less than 10% of its exponential phase value in late stationary phase. Likewise, FLAG-tagged RNase BN protein, detected by Western analysis using anti-FLAG antibody, increased rapidly and was also most abundant in exponential phase, but was reduced to barely detectable levels in late stationary phase, as indicated by the Western blot in Fig. 1B. These data indicate that expression of RNase BN is strongly dependent on growth phase, and they suggest that this enzyme functions primarily in exponential phase. The close correlation between expression of RNase BN and RNase BN protein also suggests that its regulation is most exponential phase. The close correlation between phase, and they suggest that this enzyme functions primarily in that expression of RNase BN is strongly dependent on growth indicated by the Western blot in Fig. 1 and was also most abundant in exponential phase, but was Western analysis using anti-FLAG antibody, increased rapidly to less than 10% of its exponential phase value in late stationary phase. Likewise, FLAG-tagged RNase BN protein, detected by measurement of *rbn* mRNA or RNase BN protein when compared with the 0.5-h sample, which was set at 1.0.

| Table 1: Effect of RNase BN removal on the amount of small RNAs |
|---------------------------------------------------------------|
| No. | RNA | Gene I.D. | Amount of RNA in WT | Amount of RNA in RNase BN |
|-----|-----|-----------|---------------------|--------------------------|
| 1   | MgrR| b4698     | 1.0                 | 1.0                      |
| 2   | MicA| b4414     | 1.0                 | 1.0                      |
| 3   | RffD| b4609     | 1.0                 | 1.0                      |
| 4   | SibE| b4611     | 1.0                 | 1.0                      |
| 5   | SraC| b4432     | 1.0                 | 1.0                      |
| 6   | 6S RNA| b2911 | 1.0                 | 1.0                      |
| 7   | GcvB| b4443     | 1.0                 | 1.0                      |
| 8   | GlnY| b4441     | 1.0                 | 1.0                      |
| 9   | OxyS| b4458     | 1.0                 | 1.0                      |
| 10  | RapA| b4451     | 1.0                 | 1.0                      |
| 11  | 5S RNA| Multiple | 1.0                 | 1.0                      |

* Exponential phase (Exp.) cells were harvested at \(A_{600} = 0.2\). The value for this sample was set at 1.0 for quantifying the Northern blots (supplemental Fig. S1). * Stationary phase (Sta.) cells were harvested at \(A_{600} = 3.0\). The amounts of RNAs were normalized by 5S RNA values. Gene I.D. was from the EcoGene database.

high level in exponential phase, but decreasing dramatically in stationary phase. As a consequence, 6S RNA remains low during exponential phase and is stabilized and accumulates in stationary phase cells as RNase BN disappears. *In vitro* assays revealed that RNase BN can directly cleave 6S RNA using its endoribonuclease activity. Furthermore, the 6S RNA:pRNA duplex is an even more favorable substrate than is free 6S RNA. Taken together, these findings suggest that a primary role of RNase BN in *E. coli* may be to down-regulate certain sRNAs in exponential phase cells.

TABLE 1

Effect of RNase BN removal on the amount of small RNAs

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- **Effect of RNase BN removal on the amount of small RNAs**

- **No.**

- **RNA**

- **Gene I.D.**

- **Amount of RNA in WT**

- **Amount of RNA in RNase BN**

- **Exp.**

- **Sta.**

- **Exp.**

- **Sta.**

- **1**

- **MgrR**

- **b4698**

- **1.0**

- **1.5**

- **1.0**

- **1.0**

- **1.6**

- **2**

- **MicA**

- **b4414**

- **1.0**

- **3.5**

- **1.1**

- **1.1**

- **3.5**

- **3**

- **RffD**

- **b4609**

- **1.0**

- **2.0**

- **1.2**

- **1.2**

- **2.0**

- **4**

- **SibE**

- **b4611**

- **1.0**

- **8.0**

- **2.3**

- **2.3**

- **8.0**

- **5**

- **SraC**

- **b4432**

- **1.0**

- **4.5**

- **1.0**

- **1.0**

- **4.6**

- **6**

- **6S RNA**

- **b2911**

- **1.0**

- **3.0**

- **2.0**

- **2.0**

- **3.1**

- **7**

- **GcvB**

- **b4443**

- **1.0**

- **0.6**

- **1.5**

- **0.4**

- **8**

- **GlnY**

- **b4441**

- **1.0**

- **0.6**

- **1.0**

- **0.6**

- **9**

- **OxyS**

- **b4458**

- **1.0**

- **<0.1**

- **2.1**

- **<0.1**

- **10**

- **RapA**

- **b4451**

- **1.0**

- **0.9**

- **0.7**

- **0.7**

- **0.9**

- **11**

- **5S RNA**

- **Multiple**

- **1.0**

- **1.0**

- **1.0**

- **1.0**

- **1.0**

- **15**

- **19**

- **22**

In the absence of RNase BN, the levels of four of these sRNAs were unaffected, but two of them, SibE and 6S RNA, increased ~2-fold in exponential phase, whereas remaining unchanged in stationary phase (Table 1 and supplemental Fig. S1). Examination of several additional sRNAs (Nos. 7–10) revealed that two of them, GcvB and OxyS, were also elevated during exponential phase in the RNase BN mutant, although their amounts normally decrease or remain the same in stationary phase (Table 1 and supplemental Fig. S1). These data implicate RNase BN in contributing to the levels of certain sRNAs in exponential phase cells. To examine this point in more detail, the rest of the study focuses on how RNase BN controls the abundance of 6S RNA, a known regulator of transcription by the RNA polymerase Ec*σ*20 holoenzyme (14), and an sRNA that increases in stationary phase.

Effects of RNase BN on 6S RNA Accumulation throughout Growth—To determine how RNase BN affects 6S RNA abundance, we first compared the accumulation of 6S RNA in wild type and *Δrbn* mutant cells throughout a complete growth cycle using Northern analysis (Fig. 2). When an overnight culture of wild type cells was diluted into fresh medium, pre-existing 6S RNA levels rapidly declined for about 2 h to ~30% of that in stationary phase, and then increased over time to their original level in late stationary phase (Fig. 2A). A similar overall accumulation pattern was observed in the *Δrbn* mutant strain, but in these cells, the reduction of 6S RNA during outgrowth was considerably less pronounced (Fig. 2B), implying that RNase BN participates in the removal of 6S RNA as cells resume growth. These data help to explain why the level of 6S RNA was found to be 2-fold higher in early exponential phase RNase BN- cells when compared with wild type (Table 1). The findings also indicate that RNase BN must be synthesized very rapidly in early exponential phase so that it may be available to participate in the removal of 6S RNA as cells resume growth, a conclusion in complete agreement with the dramatic increase in RNase BN levels shown in Fig. 1B.

Although there is some reduction of 6S RNA in the *Δrbn* cells as well during the first 2 h of growth, we believe that it is likely due to “dilution” of pre-existing 6S RNA that results from the increase in cell number during this period. As shown by the Northern analysis in Fig. 2C, the addition of chloramphenicol at the onset of outgrowth eliminated the reduction in 6S RNA in work (15, 19–22).
RNase BN− cells because the antibiotic inhibited cell growth, and hence, there was no dilution effect. 6S RNA was also stabilized in wild type cells in the presence of chloramphenicol because synthesis of RNase BN was blocked, leaving the cell devoid of any RNase BN. The stability of 6S RNA under these conditions suggests that RNase BN is critical for 6S RNA degradation, and that other ribonucleases already present in the cells are unable to catalyze its removal.

Additional evidence that RNase BN acts on 6S RNA in vivo comes from an experiment in which the nuclease was overexpressed in wild type cells using a high copy number plasmid. Expression of rbn from this plasmid was driven by an IPTG-inducible promoter. 6S RNA accumulation was detected by Northern analysis. As shown in Fig. 2D, the normal accumulation of 6S RNA in late exponential and stationary phase was reduced when RNase BN was overexpressed due to IPTG induction. Even in the absence of IPTG, a condition in which RNase BN would be elevated slightly due to leaky expression, there was a slower accumulation of 6S RNA. All of these findings support the conclusion that RNase BN acts on 6S RNA and reduces its level in early exponential phase cells, and then, as RNase BN levels decrease, this enables 6S RNA to accumulate as cells approach and enter stationary phase.

Stability of 6S RNA in WT and Δrbn Mutant Cells—To shed further light on the action of RNase BN on 6S RNA, we examined whether RNase BN affects 6S RNA stability. Cells in exponential phase and stationary phase were treated with rifampicin to prevent new 6S RNA synthesis, and 6S RNA levels at different times after rifampicin treatment were determined by Northern analysis. As shown in Fig. 3A, wild type exponential phase cells lost about half of their complement of 6S RNA within 20 min after rifampicin treatment, which is in agreement with an earlier work (23). In contrast, 6S RNA in RNase BN− cells was stable for at least 30 min after rifampicin treatment, indicating that the presence of RNase BN led to degradation of 6S RNA. In stationary phase cells, on the other hand, no degradation of 6S RNA was observed in either wild type or Δrbn mutant cells (Fig. 3, C and D). Based on these data, we conclude that RNase BN controls 6S RNA stability in exponential phase cells, and because RNase BN is no longer present in stationary phase cells, 6S RNA is stable irrespective of whether the cells are RNase BN− or RNase BN+. It should be noted that rifampicin also affects rRNA synthesis, which could alter the actual half-life of 6S RNA seen in this experiment, but not the difference between the two cells.

RNase BN Directly Acts on 6S RNA in Vitro—To ascertain whether RNase BN can directly cleave 6S RNA, the RNA was prepared by in vitro transcription and labeled at its 5′ end using [γ−32P]ATP and T4 polynucleotide kinase. The labeled 6S RNA was then incubated with purified wild type RNase BN or with an S273A mutant protein that is devoid of RNase activity (10). Treatment with wild type RNase BN in the presence of Mg2+ led to gradual degradation of 6S RNA, generating a 5′-terminal product ~15 nt in length (Fig. 4A, left panel, and Fig. 4B). Doubling the amount of RNase BN led to a proportional increase in the disappearance of full-length 6S RNA (Fig. 4B), suggesting normal action on the RNA substrate. In contrast, 6S RNA was unaffected by the mutant enzyme (Fig. 4C), confirming that the action on 6S RNA was, in fact, due to RNase BN.

Because 5′ labeling only revealed the 5′-terminal fragment generated by RNase BN cleavage, we also carried out primer extension analysis of cleaved 6S RNA using a primer complementary to the 3′-terminal region of 6S RNA to determine whether there might be other cleavages as well. This analysis revealed at least four additional cleavages (supplemental Fig. S2), following residues 140, 127, 92, and ~70, in addition to the cleavage that generated the ~15-nt fragment. It should be noted that the cleavage that generates the 15-nt fragment was not observed by primer extension because it is too far from the...
primer used. Nevertheless, these data show that RNase BN is able to cleave 6S RNA at multiple positions in vitro.

pRNA Increases Breakdown of 6S RNA—Upon outgrowth from stationary phase, 6S RNA is released from RNA polymerase as a 6S RNA:pRNA duplex that is rapidly degraded (14). To determine whether RNase BN also acts on this duplex, 5'-32P-labeled 6S RNA was annealed with chemically synthesized 14-nt pRNA and then the duplex was treated with purified RNase BN. As shown in Fig. 4A, right panel, RNase BN could cleave the 6S RNA:pRNA duplex, generating two products, one the same size as the product observed when free 6S RNA was cleaved by RNase BN, and a second product ~43 nt in length. Moreover, RNase BN degraded the 6S RNA:pRNA duplex more efficiently than free 6S RNA (Fig. 4B), indicating that the duplex is a more favorable substrate of RNase BN. We believe that the increased degradation efficiency is due to a rearrangement of 6S RNA structure induced by the binding of pRNA, as described previously (17).

P142G Mutant of RNase BN Works Better on 6S RNA in Vitro and Does Not Affect 6S RNA Level in Vivo—RNase BN can act as both an exoribonuclease and an endoribonuclease when it functions in the processing of tRNA precursors (9). To determine which activity of RNase BN might contribute to 6S RNA turnover, we performed an in vitro assay using the P142G mutant of RNase BN, which was shown to be devoid of the exoribonuclease activity, while maintaining the endoribonuclease activity (9). As shown in Fig. 5, A and B, the P142G mutant of RNase BN actually degraded 6S RNA more efficiently than the wild type protein, consistent with its increased endoribonuclease activity. Thus, the exoribonuclease activity of RNase BN is not required for its action on 6S RNA in vitro.

To determine whether the exoribonuclease activity of RNase BN was also unnecessary in vivo, we compared 6S RNA accumulation in the wild type and in a chromosomal P142G mutant strain using Northern analysis. As shown in Fig. 5, C and D, there was no difference in the pattern of 6S RNA degradation and accumulation between the wild type and the P142G mutant strains at different stages of growth, indicating that in vivo RNase BN acts on 6S RNA using primarily its endoribonuclease activity.
Regulation of 6S RNA by RNase BN

The data presented here reveal that the amount of RNase BN present in E. coli is regulated by the growth phase of the cells. Thus, RNase BN is present at its highest level in early to mid-exponential phase, decreasing thereafter to the point where it is essentially absent from stationary phase cells. This dramatic growth phase-dependent variation in RNase BN implies that its function may be limited to early exponential growth phase-dependent variation in RNase BN suggests that it may participate in the transition of cells from stationary to exponential phase, and this conclusion is supported by its action on 6S RNA

FIGURE 5. The exoribonuclease activity of RNase BN is not required for action on 6S RNA. A, cleavage of 6S RNA by wild type RNase BN or P142G mutant protein in vitro. 6S RNA (0.05 μM) was treated with 0.4 μM wild type and mutant RNase BN. Samples were analyzed as described under “Experimental Procedures.” The size of products was determined using a 32P-labeled mixture of five oligonucleotides. B, rate of 6S RNA disappearance upon treatment with wild type or mutant RNase BN. The 6S RNA remaining in panel A was quantified and is presented as the percentage of the initial substrate present. C, 6S RNA accumulation in wild type and a chromosomal P142G mutant strain. Overnight cultures of the two strains were diluted 1:200 into 40 ml of YT medium. RNA was isolated at the indicated time points, and 2 μg was added to each lane. Northern analysis was performed by probing for 6S RNA and 5S RNA simultaneously. The number below each lane is the relative amount of 6S RNA when compared with the 0.5 h sample, which was set at 1.0. D, quantification of the data in panel C. The amount of 6S RNA was normalized using 5S RNA as an internal reference, and the 6S RNA level at zero time in the wild type and P142G mutant strains was set at 100%. Representative experiments of in vitro assays and Northern analysis are shown.

Discussion

The data presented here reveal that the amount of RNase BN present in E. coli is regulated by the growth phase of the cells. Thus, RNase BN is present at its highest level in early to mid-exponential phase, decreasing thereafter to the point where it is essentially absent from stationary phase cells. This dramatic growth phase-dependent variation in RNase BN implies that its function may be limited to early exponential growth in E. coli. In fact, the finding that the level of RNase BN rises within 30 min of transfer of cells to new growth medium strongly suggests that it may participate in the transition of cells from stationary to exponential phase, and this conclusion is supported by its action on 6S RNA, a global transcriptional regulator. The mechanism of RNase BN regulation remains unclear, but the fact that rbn mRNA changes in concert with RNase BN protein makes it likely that there is a transcriptional component to the regulation. Moreover, the growth phase-dependent regulation of RNase BN adds to a growing list of bacterial RNases that are known to be regulated in this manner (24, 25), and to the regulation of RNases, in general (1).

RNase BN/RNase Z is known to be able to participate in tRNA maturation in E. coli, but this role only becomes obvious when multiple other processing enzymes are absent (11), a condition that only occurs by laboratory manipulation. Thus, the primary physiological function of RNase BN has remained elusive. The findings presented here indicate that RNase BN functions primarily in exponential phase, and that it affects the levels of some sRNAs, such as 6S RNA, SibE, GcvB, and OxyS, and presumably others as well. For further analysis, 6S RNA was used as the model to investigate how RNase BN acts to regulate sRNAs. 6S RNA functions as a global transcription regulator by interacting with Eor20, but the mechanism that drives its accumulation in stationary phase cells is still unclear (14). In addition to regulated transcription, degradation or stability control is also thought to be important for the variation in 6S RNA levels that occurs between cells in exponential phase and stationary phase. However, until now, no ribonuclease had been identified as being responsible for 6S RNA degradation, although RNase E, RNase G, and some exoribonucleases were known to participate in the processing of 6S RNA precursors (26–28). Here, we found that RNase BN regulates 6S RNA levels and its stability in vivo. Based on in vitro assays, this regulation appears to be accomplished by direct cleavage of 6S RNA. Additionally, in contrast to its action on tRNA precursors in which both its exoribonuclease and its endoribonuclease activities are involved (9), only the endoribonuclease activity of RNase BN is important when acting on 6S RNA.

One of the more interesting findings related to 6S RNA is that it can be used as a template by RNA polymerase to produce a small RNA, termed pRNA, that plays an important role in the transition of cells from stationary phase to exponential growth (29). The synthesis of pRNA, which can be inhibited by rifampicin, leads to the release of 6S RNA from RNA polymerase as a 6S RNA:pRNA duplex, and as a consequence, there is a rapid increase in available RNA polymerase that enables enhanced transcription for active growth (29). Our findings show that the released 6S RNA:pRNA duplex is immediately degraded by RNase BN. Moreover, as shown by in vitro assays, the 6S RNA:pRNA duplex is a more favorable substrate than free 6S RNA, ensuring its rapid degradation during outgrowth. In addition, our findings suggest that RNase BN helps to regulate the level and stability of 6S RNA in exponential phase cells. However, the dynamics of 6S RNA in exponential phase is complicated, because it includes the synthesis, degradation, and even cycling on and off RNA polymerase (29) inasmuch as most of the 6S RNA present is bound to RNA polymerase (15). Nevertheless, it is clear that RNase BN is an important contributor to the metabolism of 6S RNA in exponential phase cells.

In the RNase BN mutant, twice as much 6S RNA accumulated in exponential phase cells when compared with wild type. In contrast, the absence of either RNase E or RNase III had much less of an effect (26, 30, 31). Consequently, we propose that RNase BN is the primary ribonuclease regulating 6S RNA levels in E. coli. Additional support for this conclusion comes from our finding that although 6S RNA has a half-life of ~20 min in exponential phase cells, it is completely stable in stationary phase, a condition in which RNase BN is absent. In addition, pRNA synthesis is reduced in stationary phase cells, and this would also contribute to the stability of 6S RNA.

Taken together, the results presented here demonstrate an important physiological function for RNase BN. By controlling the amount and stability of 6S RNA, a crucial transcription regulator, RNase BN has a central role in overall cell regulation. Moreover, it is likely that other small RNAs, such as SibE and OxyS, will also be found to be affected by RNase BN. In view of
its important role in regulating small RNA regulators, understanding how RNase BN itself is regulated will be of considerable interest.

**Experimental Procedures**

**Materials—**[$\gamma$-$^{32}$P]ATP was purchased from PerkinElmer Life Sciences. T4 polynucleotide kinase and avian myeloblastosis virus reverse transcriptase were products of New England Biolabs. RQ1 RNase-free DNase and the Riboprobe® *In Vitro* Transcription System were obtained from Promega. The Thermo Sequence Cycle Sequencing Kit was from Affymetrix Inc. M-MLV reverse transcriptase was obtained from Invitrogen. The SequaGel®UreaGel system for denaturing urea-polyacrylamide gel was from National Diagnostics. Oligonucleotides and anti-FLAG M2 mAbs were obtained from Sigma. Anti-mouse IgG HRP conjugate was from Santa Cruz Biotechnology. The KOD Hot Start DNA Polymerase was purchased from Novagen. The Nytran™ SuPerCharge (SPC) nylon transfer membrane was from GE Healthcare. ExpressHyb™ Hybridization Solution was obtained from Clontech Laboratories Inc. All chemicals were reagent grade.

**Vectors, Bacterial Strains, and Growth Conditions—**The RBN expression vector, constructed in earlier work (32), was a gift from Dr. Kenneth Rugh (University of Miami). Wild type *E. coli* K-12 strain MG1655 (Seq) *rph*+ was obtained from laboratory stock. The Δbns strain and the chromosomal P142G mutagen strain of RNase BN were constructed in previous work (9). A 2×FLAG sequence was fused to the C terminus of chromosomal RNase BN by recombineering using primers F1 and F2 (see oligonucleotide sequences in supplemental Table S1), and the resulting gene fusion was confirmed by DNA sequencing. Cells were grown overnight in 5 ml of YT medium (5 g/L yeast extract, 8 g/L tryptone, 5 g/L NaCl) at 37 °C. Cultures were diluted 1:200 into fresh YT medium, and equal amounts were removed at various time points and immediately mixed with ice-cold stop solution (95% ethanol, 5% phenol). Antibiotics, when present, were at the following concentrations: kanamycin, 25 μg/ml; ampicillin, 100 μg/ml. To inhibit the outgrowth, overnight cultures were diluted 1:200 into 37 °C pre-heated YT medium and incubated at 37 °C for 2 min, followed by the addition of chloramphenicol (final concentration is 200 μg/ml).

**RNA Decay—**Overnight cultures were diluted 1:200 into fresh YT medium and grown to exponential phase (A$_{600}$ = 0.4) or stationary phase (A$_{600}$ = 3.6). Rifampicin was then added to a final concentration of 500 μg/ml. After 75 s to allow complete inhibition of new transcription, cells were removed for the zero time sample. Additional samples were removed at different time points as indicated in the figure legends. Changes in 6S RNA levels were determined by Northern analysis.

**RNA Extraction, RT-PCR, and Northern Analysis—**Cellular total RNA was prepared using the hot phenol method described previously (33), and the RNA concentration was determined by UV spectroscopy. Genomic DNA in the RNA samples was removed using RQ1 RNase-free DNase I. DNase I-treated RNA (2 μg) was reverse-transcribed with an *rph* gene-specific primer, 5′-CTGGTATACAGGGGCAGGTTTCTC-3′, using M-MLV reverse transcriptase. The resulting cDNA was amplified by PCR using the forward primer, 5′-ACTGCATAACCGC-CTTTAAC-3′, and the reverse primer, 5′-ATGACATCGAC-ACCTTTAGC-3′. PCR products were run on a 1.0% agarose gel and visualized using the BioDoc-It imaging system (UVP). Northern analysis of 5S RNA and 6S RNA was performed by fractionating 2 μg of RNA on 6% polyacrylamide/7.5 × urea gels. RNA was then transferred onto nylon membranes by the semi-dry transfer method. Membranes were fixed by UV irradiation and incubated overnight with probes for 6S RNA (5′-GAATCTCCGAGATGCCGCCG-3′) and 5S RNA (5′-ATG-CCTGCGATTTCTACTCTCGC-3′) together. For the Northern analyses of SibE, MgrR, SraC, RyfD, and MicA (see oligonucleotide sequences in supplemental Table S1), 15 μg of RNA was added and transferred onto the nylon membrane. The membrane was first probed for 5S RNA, and then stripped and reprobed for appropriate oligonucleotides. All probes were 5′-end-labeled using [$\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase. The blots were visualized using a STORM 840 phosphorimaging device. ImageQuant software was used to quantify the bands.

**Western Blotting—**Whole cell extracts were prepared as described (15). Protein concentration was measured by the Bradford assay. Equal amounts of protein (20 μg) were separated on 10% SDS-PAGE gels and transferred onto PVDF membranes. Membranes were then treated with 3% nonfat milk and probed with anti-FLAG M2 mAbs (1:1,000 dilution). RNase BN was detected with HRP-conjugated anti-mouse IgG antibody and visualized with ECL solutions.

**Substrate Preparation—**Templates for in vitro transcription of 6S RNA were amplified by PCR from genomic DNA of strain MG1655 (Seq) *rph*+ using the forward primer, 5′-TAAATAC-GACTCATAATGGGAGATTTCTGATAGTGGC-AAG-3′, and the reverse primer, 5′-GAATCTCCGAGATGCCGCCG-3′. PCR products were purified according to manufacturer’s instructions for the GenElute™ PCR Clean-up Kit (Sigma-Aldrich). In vitro transcription was performed using T7 RNA polymerase according to instructions provided with the Riboprobe® *in vitro* transcription system. The reaction mixture was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1), and 6S RNA products were precipitated overnight with ethanol at -20 °C. Samples were centrifuged at 20,000 × g for 15 min at 4 °C, and the resulting pellets were washed with ice-cold 75% ethanol. The RNA pellets were air-dried and dissolved in 50 μl of RNase-free water.

**Purification of 6S RNA—**was performed by annealing with calf intestine alkaline phosphatase to remove its 5′-terminal phosphate, and the 5′-end was subsequently labeled using [$\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase. 32P-labeled 6S RNA in 1× reaction buffer (70 mM Tris-HCl, pH 7.6, 10 mM MgCl$_2$, 5 mM DTT) of T4 polynucleotide kinase was finally obtained by removing the unincorporated radioactivity using a NuCaw™ spin column (Thermo Fisher). The 14-nucleotide-primer (5′-AUCGCUCAGGGCGGGA-3′), described in previous work (29), was synthesized by Sigma. To prepare the 6S RNA:primer duplex, a 12.5-μl mixture containing 2.5 μl (1.25 pmol) of 32P-labeled 6S RNA, 1 μl (1.5 pmol) of primer, and 9 μl of 1× Tris-EDTA was heated at 95 °C for 5 min followed by slow cooling to room temperature. For comparison, 2.5 μl (1.25 pmol) of 32P-labeled 6S RNA alone was treated in parallel in 10 μl of 1× Tris-EDTA.
In Vitro Assays of RNase BN—Purification of wild type RNase BN, the S273A mutant protein, and the P142G mutant protein was described previously (9, 10). Cleavage of 6S RNA or the 6SRNA:pRNA duplex was performed in a 30-μl reaction mixture containing 0.2 or 0.4 μM purified protein, 0.01 or 0.05 μM substrate, and 1× reaction buffer (10 mM Tris-HCl, pH 7.5, 200 mM potassium acetate, 5 mM MgCl₂). After incubation for the indicated times, reactions were stopped by adding 2 volumes of gel loading buffer (90% formamide, 20 mM EDTA, 0.05% SDS, 0.025% bromphenol blue, and 0.025% xylene cyanol). Products were separated on 8% polyacrylamide/7.5M urea gels and quantified using a STORM 840 phosphorimaging device.

Primer Extension Analysis to Identify Cleavage Sites on 6S RNA—To prepare templates for primer extension analysis, 0.1 μM unlabeled 6S RNA was cleaved by 1 μM purified RNase BN in a 100-μl reaction mixture using 1× reaction buffer containing Mg²⁺. Portions were removed at 0, 10, 20, 40, and 60 min after incubation at 37 °C, followed by extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitation with ethanol prior to primer extension. Primer (5′-GAATCTCCCG-AGATGCCGCCG-3′) positioned at residues 164–183 of 6S RNA was 5′ end-labeled using [γ-³²P]ATP and T4 polynucleotide kinase. The labeled primer was used for the sequencing reactions and primer extension analysis. Cleaved 6S RNA was mixed with labeled primer and dNTPs, heated to 65 °C for 5 min, and slowly cooled to 42 °C, followed by the addition of 2 μl of 10× reaction buffer and 10 units of avian myeloblastosis virus reverse transcriptase. Samples were incubated at 42 °C for 1 h, and reactions were terminated by the addition of 20 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromphenol, 0.05% xylene cyanol FF). The sequencing reactions were performed according to the manufacturer’s instructions for the Thermo Sequence Cycle Sequencing Kit. Products were resolved on 8% polyacrylamide/7.5 M urea gels in parallel with sequencing reaction samples. Dried gels were exposed to X-film for 3 h.

Author Contributions—H. C., T. D., and M. P. D. designed the experiments. H. C. performed the experiments. H. C. and M. P. D. analyzed the data and wrote the paper.

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