A novel mechanism of ribonuclease regulation: GcvB and Hfq stabilize the mRNA that encodes RNase BN/Z during exponential phase

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RNase BN, the Escherichia coli RNase Z family member, plays a limited role in tRNA metabolism, in contrast to most other organisms. However, RNase BN does act on 6S RNA, the global transcription regulator, degrading it in exponential-phase cells and maintaining it at low levels during this phase of growth. RNase BN levels decrease in stationary-phase cells, leading to elevation of 6S RNA and subsequent regulation of RNA polymerase. These findings were the first indication that RNase BN itself is growth phase–regulated. Here, we analyze the mechanism of this regulation of RNase BN. We find that RNase BN decreases in stationary phase because its mRNA becomes unstable, due primarily to its degradation by RNase E. However, in exponential-phase cells rbn mRNA is stabilized due to binding by the sRNA, GcvB, and the protein, Hfq, which reduce cleavage by RNase E. Because the amount of GcvB decreases in stationary phase, rbn mRNA is less protected and becomes increasingly unstable resulting in reduction in the amount of RNase BN. The small RNA-dependent, positive regulation of RNase BN in exponential-phase cells is the first example of this novel mechanism for RNase regulation.

Ribonucleases (RNases) are integral components of every aspect of RNA metabolism playing a central role in maturation and degradation of all RNA molecules (1, 2). However, RNases may also carry out detrimental reactions leading to the unwanted destruction of important RNAs (3). As a consequence, cells have evolved regulatory processes to control the amount and activity of individual RNases through mechanisms that have begun to be identified in recent years (3–5). In addition, it has become apparent that cells also regulate the levels of specific RNases in response to changes in environmental conditions (6). Understanding the diverse mechanisms that underlie the processes that regulate RNases is of critical importance in light of the central role of these enzymes in RNA metabolism.

RNase BN is both an endoribonuclease and a 3’ → 5’-exoribonuclease, and it is the Escherichia coli member of the RNase Z family of RNases (7–9). It was originally discovered based on its requirement for the maturation of certain phage T4 tRNA precursors that lack an encoded 3’-terminal -CCA sequence (10, 11), a common feature of the specificity of RNase Z family members. This raised the question of why E. coli contains an RNAse Z homologue as all its tRNA genes already encode the -CCA sequence (12), and it strongly suggested that RNase BN serves a different function than tRNA maturation in this organism. Recently, we found that RNase BN controls the level of the global transcription regulator, 6S RNA, throughout growth of E. coli (13). We observed that 6S RNA remains low in exponential-phase cells because it is degraded by RNase BN and that it increases in stationary phase as the amount of RNase BN decreases (13). In addition to their importance for 6S RNA metabolism, these findings also indicated that RNase BN itself is subject to growth phase-dependent regulation.

In this paper, we identify the unusual mechanism by which RNase BN is regulated. We find that the rbn message and RNase BN decrease in stationary phase as much as 10-fold from their levels in exponential-phase cells. The reduction is not due to a transcriptional effect because that process appears to be unchanged in the two phases of growth. Rather, it results from the fact that the rbn message is much less stable in stationary-phase cells. RNase E is largely responsible for the instability of rbn mRNA, although RNase G can also contribute to the message decay. RNase BN protein is stable during both phases of growth, supporting the conclusion that the lower amount of RNase BN in stationary-phase cells is a consequence of the greater instability of its message. Analysis of factors that might affect rbn mRNA stability revealed that the protein, Hfq, and the sRNA, GcvB, act to stabilize the exponential-phase message; however, as the level of GcvB decreases in stationary-phase cells, rbn mRNA becomes increasingly sensitive to degradation, ultimately leading to reduction in the amount of RNase BN. In vitro analysis using purified components directly confirmed that GcvB binds to rbn mRNA in the presence of Hfq and that RNase E cleaves rbn mRNA. These data identify a novel mechanism for regulation of RNase BN and add to the growing list of diverse processes that control ribonucleases.
Regulation of RNase BN/Z

Figure 1. Accumulation of RNase BN and rbn mRNA throughout growth. Overnight cultures were diluted into 50 ml of YT medium and grown at 37 °C. Cells were removed at the indicated time points followed by preparation of total RNA or protein. A, accumulation of RNase BN protein analyzed by immunoblotting. Twenty μg of total protein was separated by 10% SDS-PAGE and subjected to Western blot analysis using anti-FLAG primary antibody as described under "Experimental procedures," and as shown in our previous work (13). B, accumulation of rbn mRNA determined by RT-qPCR. cDNA was synthesized from 1.2 μg of total RNA and subjected to RT-qPCR as described under "Experimental procedures." The amount of rbn mRNA was normalized by comparison with RT-qPCR of 16S RNA at each time point. The relative amount of RNase BN or rbn mRNA is presented compared with the corresponding 1-h sample, which was set at 1.0. The average ± S.D. from two independent experiments is presented.

Results

RNase BN is regulated by growth phase

In earlier work (13), we found that RNase BN levels decrease as cells enter stationary phase. More detailed examination of the phenomenon (Fig. 1A) revealed that reduction in the amount of RNase BN begins in late-exponential phase and that the overall decrease in RNase BN from its maximal level in exponential phase can be as much as 10-fold. Analysis of the amount of rbn mRNA (Fig. 1B) indicated that it also varied with growth phase in the same manner as observed for RNase BN protein. These data suggest that regulation of RNase BN is determined at the transcriptional or post-transcriptional level, rather than on translation or protein stability.

rbn mRNA transcription is the same in both growth phases

To examine how the amount of rbn mRNA might be regulated in exponential- and stationary-phase cells, we first determined the transcription start site in the two phases of growth using 5′-RACE (Fig. 2). Based on this analysis (Fig. 2B), transcription of the rbn message initiated at the same position in both exponential- and stationary-phase cells, at an A residue located 26 nucleotides upstream of the likely initiator ATG codon (Fig. 2A). Examination of the nucleotide sequence upstream of the transcription start site identified putative −10 and −35 promoter sequences that were in agreement with those predicted from global transcription analysis (14, 15).

To further analyze rbn transcription during the two phases of growth and to examine any effects of termination, we deleted the 3′UTR beginning at the 3rd residue following the TAA termination codon. Examination of rbn expression revealed no difference between the WT and the Δ3′UTR strain in either exponential or stationary phase (Fig. 2C). In addition, a promoter fusion between the −69 to +65 region of rbn and lacZ showed little difference in β-gal activity throughout growth (Fig. 2D). Based on these analyses, there is no indication that transcription of rbn differs between exponential- and stationary-phase cells.

rbn mRNA stability differs in the two growth phases

In light of the above findings, we sought other explanations for the dramatic reduction of rbn message in stationary-phase cells. To this end, we examined rbn mRNA stability during the two phases of growth. As shown in Fig. 3A, stationary phase rbn mRNA was much more unstable than its counterpart in exponential-phase cells, exhibiting a half-life of ~2 min compared with ~8 min for the exponential-phase message. In contrast, there was no difference in the stability of a control message encoding another RNase, that for polynucleotide phosphorylase (pnp) (Fig. 3A), indicating that instability was not a common feature of RNase mRNAs in stationary phase. Analysis of the half-life of RNase BN itself indicated that the protein was stable during both phases of growth (Fig. 3B). Based on these data, we conclude that the relative instability of rbn mRNA is a major contributor to its reduction in stationary-phase cells.

RNase E is primarily responsible for degradation of rbn mRNA

The instability of rbn mRNA in the stationary phase raised the question of why the message was so unstable compared with that in the exponential phase. To help answer this question, we first examined which RNase(s) might be responsible for degradation of the rbn mRNA. As can be seen in Fig. 4A, removal of the endoribonuclease, RNase E, led to dramatic stabilization of the rbn message, increasing its half-life from ~2 to ~10 min. However, mRNA decay still continued, although at a reduced rate suggesting the involvement of another RNase as well. In addition, as shown in Fig. 4B, the action of RNase E on the rbn message does not require its association with the RNA degradosome because deletion of the C-terminal region of RNase E, which is responsible for its association in the degradosome, had no effect on rbn mRNA degradation.

To examine which other RNase might participate in rbn mRNA degradation, we removed RNase G, a homologue of RNase E. Its removal did not affect rbn mRNA decay (Fig. 4C).
However, in the absence of both RNase E and RNase G, rbn mRNA was essentially stable in stationary-phase cells (Fig. 4D). These data indicate that RNases E and G, and primarily RNase E, are responsible for degradation of the rbn mRNA in stationary-phase cells.

Hfq affects rbn mRNA stability in exponential phase

The major difference in rbn mRNA stability between exponential- and stationary-phase cells raised the question of whether trans-acting factors might be differentially affecting the message in the two phases of growth. To address this point,
we eliminated several factors known to affect mRNA stability and determined whether their removal affected \textit{rbn} mRNA. We first examined polyadenylation catalyzed by poly(A) polymerase. As is shown in Fig. 5, A and D, mutation of the \textit{pcnB} gene encoding poly(A) polymerase (16, 17) did not affect the stability of \textit{rbn} mRNA in either exponential or stationary phase indicating that polyadenylation is not involved in the differential stability of this message. Removal of RNA pyrophosphohydrolase (RppH) by mutation of the \textit{rppH} gene (18) led to stabilization of the \textit{rbn} message in both exponential and stationary phase (Fig. 5, B and E); however, because there was no significant difference in the effect of RppH removal during the two phases of growth, it is unlikely that RppH plays any role in the differential stability of the \textit{rbn} message in exponential and stationary phase. Stabilization of mRNA upon removal of RppH is to be expected as this enzyme facilitates mRNA degradation dependent on RNase E (18).

In contrast to the above findings, removal of the RNA chaperone, Hfq, differentially affected the stability of \textit{rbn} mRNA in the two phases of growth (Fig. 5, C and F). In stationary-phase cells, \textit{rbn} mRNA was equally unstable irrespective of whether Hfq was present or not (Fig. 5F). However, in exponential-phase cells, removal of Hfq dramatically destabilized the \textit{rbn} message, reducing its half-life from \(~7\) to \(~1\) min (Fig. 5C) to a level similar to that seen in stationary-phase cells (Fig. 5F). These data suggest that the presence of Hfq stabilizes \textit{rbn} mRNA in exponential phase, but not in stationary-phase cells, and as a consequence, the mRNA has different stability during the two phases of growth.

\textbf{Hfq elevates the amount of RNase BN in exponential phase}

To further examine the role of Hfq, we determined how its removal affected the actual amount of \textit{rbn} mRNA and RNase BN. As shown in Fig. 6A, the absence of Hfq reduced the amount of \textit{rbn} mRNA in exponential-phase cells to 34\% that in WT cells (compare at \(A_{600}=0.5\)). Concomitantly, the amount of RNase BN in the exponential phase was also reduced to \(~30\%\) that in WT cells (Fig. 6B). These findings confirm that the presence of Hfq is important for maintaining RNase BN at higher levels in exponential phase compared with stationary-phase cells.

\textbf{Small RNAs play a role in maintaining RNase BN levels}

Hfq is known to promote binding of sRNAs to complementary mRNAs (19), which raised the possibility that stabilization of the exponential phase \textit{rbn} message by Hfq might also involve
an sRNA molecule that associates with rbm mRNA. This led us to examine whether any known sRNAs contained sequences that would lead them to bind tightly to rbm mRNA and were also present at higher levels in the exponential phase than in the stationary phase, a requirement for a molecule expected to stabilize the exponential phase rbm message. The results of this analysis, using IntaRNA (20), are shown in Table 1. Multiple sRNAs were found to fit the first criterion of having the potential to bind tightly to rbm mRNA. In addition, several are present at higher levels in exponential-phase cells than in stationary-phase cells. None of these interactions had been predicted previously, although very recently rbm mRNA was found among messages pulled down in a GcvB MAPS experiment (21). Four of the sRNAs (RyjB, GcvB, GlmZ, and Spf), which are present at

| sRNA | Size | Position of base pairing on rbm mRNA | Energy $^b$ kcal | Expression $^c$ | Refs. |
|------|------|--------------------------------------|------------------|----------------|-------|
| RyjB | 90   | 796–818                              | −15.7            | E > S          | 43    |
| RseX | 91   | 581–613                              | −15.6            | Unknown        | 44    |
| GcvB | 205  | 76–125                               | −13.3            | E > S          | 24    |
| GlmZ | 172  | 254–282                              | −12.6            | E > S          | 24    |
| RprA | 105  | 1055–1097                            | −11.8            | S > E          | 24, 45|
| RygB | 121  | 496–544                              | −9.6             | S > E          | 45    |
| SraB | 169  | 52–98                                | −9.2             | S > E          | 24    |
| SroB | 82   | 29–44                                | −8.5             | S > E          | 46    |
| Spf  | 109  | 582–594                              | −7.8             | E > S          | 47    |

$^a$ Transcription start site is position 1. Coding region begins at position 27.

$^b$ Position of base pairing and the energy of rbm-sRNA hybrid are predicted by IntaRNA.

$^c$ Expression is based on published data. E is exponential phase, and S is stationary phase.

Figure 5. Examination of protein factors that affect rbm mRNA stability. A–C, effect of PAPI, RppH, and Hfq on rbm mRNA stability in exponential phase (Exp.). D–F, effect of PAPI, RppH, and Hfq on rbm mRNA stability in stationary phase (Sta.). Overnight cultures of the different strains were diluted into 50 ml of YT medium and grown to exponential phase ($A_{600} = 0.4$) or stationary phase ($A_{600} = 2.0$). Cells were treated with 500 mg/liter rifampicin and harvested at the indicated time points. The relative amount of rbm mRNA compared with that at the time of rifampicin addition was determined by RT-PCR as described under "Experimental procedures" using 1.2 µg of total RNA. The average ± S.D. from two independent experiments is presented.

Figure 6. Effect of Hfq on the accumulation of rbm mRNA and RNase BN. Overnight cultures were diluted into 100 ml of YT medium, and cells were harvested at the indicated time points. Total RNA or protein was prepared as described under "Experimental procedures" and subjected to RT-PCR or immunoblotting analysis. A, accumulation of rbm mRNA in WT and Δhfq strains. cDNA was synthesized using 1.2 µg of total RNA for RT-PCR. Equal amounts of total RNA were added to each lane. B, accumulation of RNase BN in WT and Δhfq strains. Twenty µg of protein was separated by 10% PAGE and subjected to immunoblotting analysis. Coomassie Blue staining was used for the loading control. A representative experiment carried out twice with essentially identical results is shown. The relative amount of rbm mRNA or RNase BN protein is indicated with an average ± S.D. from the two biological replicates by comparing to the 2-h sample, which was set at 100.
higher levels in the exponential phase, and RseX, which displays high complementarity to \( rbn \) mRNA, were chosen for study in more detail. Each of the RNAs was eliminated by mutation, and the effect of their removal on the amount of exponential phase \( rbn \) mRNA was determined. The absence of RseX or Spf had essentially no effect, although removal of RyjB or GlmZ had a small effect, lowering the amount of \( rbn \) mRNA present by \( \approx 20\% \) compared with the parental strain containing the sRNAs (Fig. 7A).

However, the absence of GcvB led to a major effect on RNase BN. As shown in Fig. 7B, the absence of GcvB led to an \( \approx 50\% \) reduction in exponential-phase cells in the amount of \( rbn \) mRNA compared with WT, somewhat less than that seen upon removal of Hfq. In stationary-phase cells, the level of \( rbn \) mRNA was unaffected by removal of GcvB, and it remained below 20% that in WT exponential-phase cells (Fig. 7B). Moreover, the absence of GcvB reduced \( rbn \) mRNA half-life from \( >5 \) to \( 2 \) min (Fig. 7C). The absence of GcvB also resulted in a reduction in the amount of RNase BN in exponential phase (Fig. 7D), slightly less than that observed in the \( \Delta hfq \) strain (Fig. 6B).

These data strongly support the conclusion that GcvB works in concert with Hfq to stabilize \( rbn \) mRNA in exponential-phase cells, leading to a higher level of RNase BN.

Because the reduction of RNase BN in the \( \Delta gcvB \) strain was not quite as much as that in the \( \Delta hfq \) strain, we inquired whether removal of additional sRNAs might enhance the effect. To examine this point, we generated a strain lacking RyjB and GlmZ, in addition to GcvB (termed \( \Delta3 \)), as removal of these two sRNAs did lead to a small reduction of \( rbn \) mRNA (see above). As shown in Fig. 7D, the amount of RNase BN was reduced even further in the \( \Delta3 \) strain, to a level essentially the same as obtained upon removal of Hfq (Fig. 6A). This finding suggests that other sRNAs also may contribute to stabilization of \( rbn \) mRNA in exponential phase.

### GcvB levels decrease in stationary-phase cells

The data presented indicate that Hfq and GcvB together stabilize \( rbn \) mRNA in exponential-phase cells thereby maintaining RNase BN at higher levels during this phase of growth. A corollary of this conclusion is that Hfq, GcvB, or both should

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**Figure 7. Effect of GcvB on \( rbn \) mRNA and RNase BN.**

*Panel A:* effect of four small sRNAs (RyjB, GlmZ, Spf, and RseX) on the accumulation of \( rbn \) mRNA. Overnight cultures of wildtype (WT), \( \Delta hfq \), and \( \Delta gcvB \) strains were diluted into 50 ml of YT medium. Cells were removed at the indicated growth stages monitored by absorbance measurement at 600 nm (exp, \( A_{600} = 0.5 \); sta, \( A_{600} = 2.0 \)). The relative amount of \( rbn \) mRNA was determined by RT-PCR using 1.2 \( \mu \)g of total RNA, and it was quantified by setting the WT exponential phase sample to 100. The average ± S.D. from two independent experiments is presented.

*Panel C:* effect of GcvB on the stability of \( rbn \) mRNA. Overnight cultures of WT and \( \Delta gcvB \) strains were diluted into 50 ml of YT medium and grown to exponential phase (\( A_{600} = 0.5 \)). Cells were treated with 500 mg/liter rifampicin, and samples were removed at the indicated time points. The amount of \( rbn \) mRNA was determined by RT-PCR using 1.2 \( \mu \)g of total RNA. The average ± S.D. from two independent experiments is presented.

*Panel D:* accumulation of RNase BN in the \( \Delta gcvB \) strain and the triple knockout strain (\( \Delta3 \)) lacking GcvB, RyjB, and GlmZ. Cells containing chromosomal FLAG-tagged \( rbn \) were harvested at the indicated time points. Twenty \( \mu \)g of total protein was separated by 10% SDS-PAGE and subjected to Western blotting using anti-FLAG primary antibody. Coomassie Blue staining was used as the loading control. A representative experiment carried out twice with essentially identical results is shown. The number below each lane is the relative amount of RNase BN protein when compared with the WT sample at \( A_{600} = 0.5 \), which was set at 100.
decrease in stationary-phase cells, and this is what renders \textit{rbn} mRNA more unstable leading to the lower amount of RNase BN. Data in the literature have been unclear on Hfq levels in the two phases of growth (22, 23), whereas GcvB is thought to be present at decreased levels in stationary phase (24). Therefore, it was deemed important to determine the levels of the two factors in our strains under our growth conditions. To this end, we analyzed the amount of Hfq and GcvB throughout growth. These data are presented in Fig. 8.

As shown in Fig. 8A, we find that Hfq levels increased about 2-fold as cells progressed from early exponential phase through stationary phase. In contrast, GcvB first increased in exponential phase, but then decreased dramatically (as much as 7-fold) in stationary phase. In contrast, GcvB first increased in exponential phase, but then decreased dramatically (as much as 7-fold) in stationary phase. These data are presented in Fig. 8.

\textbf{GcvB binds \textit{rbn} mRNA}

The data presented are most consistent with the conclusion that Hfq and GcvB bind to \textit{rbn} mRNA and protect against cleavage by RNase E due to the complementarity of GcvB to various regions of the \textit{rbn} message (Fig. 9, A–C). Three regions of complementarity were identified with the strongest binding expected to be in the region between residues 76 and 125 of \textit{rbn} mRNA (Fig. 9C) within the coding region of the message. To directly determine whether GcvB binds to \textit{rbn} mRNA, a gel-shift analysis was carried out. Purified GcvB was incubated with a 5'-labeled fragment of \textit{rbn} mRNA encompassing residues 1–165 that contains the regions complementary to GcvB (Fig. 9D). As can be seen, GcvB, by itself, did not interact with the \textit{rbn} message under these assay conditions (lanes 2–4), although binding could be observed at 10-fold higher concentrations of \textit{rbn} mRNA (Fig. 9E), indicating a direct interaction between the two RNAs. Upon addition of Hfq, under the conditions of Fig. 9D, two slower migrating bands of \textit{rbn} mRNA were observed, one that formed in the presence of Hfq alone (lane 5) and a second band generated in the presence of both Hfq and GcvB (lanes 6–8). The latter band increased with increasing amounts of GcvB, whereas the amount of the \textit{rbn} mRNA–Hfq binary complex decreased, suggesting that addition of GcvB converts the binary complex to one containing all three components. This conclusion was supported by destruction of both complexes upon incubation with proteinase K (Fig. 9D, lanes 9–12), indicating that each complex contained Hfq. Interestingly, the binary complex of \textit{rbn} mRNA and Hfq migrates more slowly than the ternary complex that also contains GcvB. This observation suggests that Hfq, by itself, may bind at multiple positions on the message and that the addition of GcvB narrows the binding to regions of complementarity between the mRNA and the sRNA. Moreover, these data also show that Hfq greatly stimulates binding of GcvB to the \textit{rbn} mRNA.

\textbf{RNase E cleaves \textit{rbn} mRNA}

To directly determine whether RNase E cleaves the \textit{rbn} message and to identify the positions of cleavage, purified RNase E was incubated with the 165-nt \textit{rbn} mRNA fragment, and the products were analyzed by primer extension (Fig. 9F). RNase E cleaved the mRNA fragment at multiple positions along the mRNA sequence. Three of the cleavages, at nt 85, 96, and 103, were within the region of greatest complementarity to GcvB (Fig. 9C). Strong, longer extension products (noted by arrows in Fig. 9F) also were observed, indicating that additional cleavages closer to the 5’ end of the message also occur \textit{in vitro}, but their exact positions could not be determined by this method due to poor resolution of the ladder in this region of the gel. To look more closely at these cleavages, the 5’-32P-labeled mRNA fragment was used as the substrate, and the products were analyzed by gel electrophoresis (Fig. 9G). Multiple bands were observed, confirming that RNase E cleaves at several positions near the 5’ end of the message fragment (Fig. 9G, lane 2). It should be noted that this analysis only detects cleavage products that retain the labeled 5’ end. Nevertheless, the data in Fig. 9, F and G, clearly indicate that RNase E cleaves \textit{rbn} mRNA at multiple specific sites.

The analysis in Fig. 9G could also be used to assess whether GcvB and/or Hfq protected the mRNA fragment against RNase E cleavage. Addition of GcvB had no effect on cleavage by RNase E (Fig. 9G, lane 3), but the presence of Hfq alone (lane 4) or Hfq and GcvB (lane 5) completely protected the mRNA.

\textbf{Figure 8. Accumulation of Hfq and GcvB throughout growth.} A, accumulation of Hfq. Overnight cultures of the WT strain carrying chromosomal 3×FLAG-tagged \textit{hfq} were diluted into 100 ml of fresh YT medium, and cells were removed at the indicated time points. Ten \( \mu \text{g} \) of total protein was resolved by 15% SDS-PAGE and subjected to immunoblotting using antibody directly against the FLAG tag. Coomassie Blue staining was used as the loading control. B, accumulation of GcvB RNA. Fifteen \( \mu \text{g} \) of total RNA was separated by 6% urea-PAGE and subjected to Northern blot analysis using probes specific for GcvB and 5S RNA. A representative experiment carried out twice with essentially identical results is shown. The relative amount of Hfq or GcvB is indicated with average ± S.D. from the two biological replicates by comparing to the sample at \( A_{600} = 0.2 \), which was set at 1.0.
Regulation of RNase BN/Z

Figure 9. In vitro interaction of GcvB with rbn mRNA and cleavage by purified RNase E. A–C, predicted base pairing between GcvB and three regions of the 5′-165-nt fragment of rbn mRNA using the IntaRNA program. Numbering of nucleotides on rbn mRNA and GcvB is relative to the transcription start site of each RNA. Arrows indicate sites of cleavage by RNase E determined by the primer extension assay shown in E, D, interaction of GcvB with rbn mRNA. 5′-Labeled rbn fragment (0.1 pmol) and 0.4 pmol (lanes 2, 6, and 10), 0.8 pmol (lanes 3, 7, and 11), or 1.6 pmol (lanes 4, 8, and 12) of GcvB were incubated with or without purified Hfq in a 10-μl reaction. For reactions in lanes 9–12, 10 μg of protease K was added following complex formation to remove Hfq. Samples were analyzed on 5% native polyacrylamide gels. A representative experiment carried out twice with essentially identical results is shown. E, interaction of GcvB with rbn mRNA in the absence of Hfq, 5′-Labeled rbn fragment (1 pmol) was incubated with 1, 2, 4, 8, or 16 pmol of GcvB (lanes 2–6, respectively) in a 20-μl reaction. F, analysis of RNase E cleavage sites on rbn mRNA. The rbn fragment (1–165) was synthesized by in vitro transcription and digested with purified RNase E (N-terminal protein, 1–498). Digestion products were subjected to primer-extension analysis as described under “Experimental procedures.” Arrows indicate all time-dependent cleavage products. The positions of the three cleavage sites within the region of highest complementarity to GcvB are numbered based on the sequence ladders. G, effect of GcvB and Hfq on in vitro cleavage of rbn mRNA by RNase E. Reaction mixtures were set up as described under “Experimental procedures.” Reaction mixtures lacking RNase E were first incubated for 20 min at 37 °C, followed by addition of RNase E and additional incubation for 15 min. Cleavage products were analyzed by 8% urea-PAGE. A mixture of 5′-labeled DNA oligonucleotides was used to evaluate the size of cleavage products. D–G, representative experiments carried out twice with essentially identical results are shown.

Unfortunately, because Hfq binds strongly to the message in vitro by itself (Fig. 9D), and thereby protects against RNase E action (Fig. 9G, lane 4), we were unable to confirm the role of GcvB identified in vivo by this in vitro analysis. Nevertheless, taken together these data confirm the in vivo findings that GcvB binds to rbn mRNA and that the message is a substrate of RNase E.

Discussion

The studies presented here provide a clear explanation for the growth phase-dependent reduction of RNase BN as cells progress from exponential to stationary phase. Thus, we found the following: 1) rbn mRNA is present at lower levels in stationary-phase cells and that this reduction is due to greater instability of the message at this stage of growth; 2) primarily RNase E, but also RNase G, are responsible for rbn mRNA degradation; 3) the sRNA, GcvB, and the protein Hfq stabilize rbn mRNA in exponential-phase cells, and this stabilization can be reversed upon elimination of either of these factors by mutation; 4) GcvB binds and RNase E cleaves rbn mRNA in vitro; and 5) GcvB levels normally decrease in stationary-phase cells that leads to the instability of rbn mRNA and ultimately to the reduction in RNase BN. This regulatory process joins the regulation of RNase R by tmRNA (25) as the second example in which an sRNA regulates an RNase. However, in the former case, regulation was at the level of RNase R protein stability (25), rather than on mRNA stability, as described here. These findings further emphasize the importance of RNase regulation both for limiting unwanted deleterious reactions and for altering RNase activity in response to changes in environmental conditions.

Although GcvB was the primary small RNA to affect rbn mRNA stability, it is likely that other sRNAs may also contribute to some degree. First, removal of RyjB or GlmZ, two other sRNAs with complementarity to rbn message, led to ~20% reductions in rbn mRNA in exponential phase; and second, elimination of RyjB and GlmZ, in addition to GcvB, resulted in a greater reduction in the amount of RNase BN in exponential-phase cells (Fig. 7C). Inasmuch as Hfq acts together with multiple sRNAs, the finding of a greater reduction of RNase BN upon its elimination than that of GcvB alone also supports the involvement of other sRNAs in stabilizing rbn mRNA. We do not yet understand why multiple sRNAs would each regulate rbn mRNA stability, but it raises the possibility that diverse, independent signals can affect RNase BN levels. Further work will be necessary to clarify this point.

Hfq mediates many interactions between sRNAs and mRNAs leading to a wide range of negative and positive regulatory effects on the mRNA targets (19). To date, only a handful.
of examples have been identified in which binding of a small RNA has been found to stabilize an mRNA against RNase action (26, 27), and this is the only example in which the target mRNA itself encodes an RNase. Inasmuch as the RNase product, in this case RNase BN, has the potential to affect many RNA molecules, this regulatory process could be of widespread significance to cellular metabolism. In fact, we have already found that RNase BN regulates 6S RNA abundance keeping its level low in exponential-phase cells, but allowing it to increase in stationary phase (13). Because 6S RNA is an important regulator of σ70-containing RNA polymerase, acting to reduce its activity in stationary-phase cells (28), the regulation of RNase BN could have significant consequences for gene expression.

It is somewhat surprising that GcvB is involved in target activation of RNase BN. GcvB is an important sRNA that targets a large number of genes in bacteria (29), including many involved in amino acid metabolism (30). Global transcription analysis revealed that the amounts of most known GcvB targets are higher in stationary phase than in exponential phase (31), implying that GcvB most commonly acts as a repressor of gene expression in exponential-phase cells, and that this repression ceases as GcvB levels decrease in stationary phase. Hence, its action on RNase BN in which it stabilizes rbn mRNA and thereby elevates the RNase in exponential phase is quite unusual. Interestingly, GcvB itself is stabilized and regulated by Hfq (32), but whether this plays any role in the reduction in GcvB in stationary phase is not known. Nevertheless, the intertwined regulatory circuits that encompass RNase BN, 6S RNA, σ70-RNA polymerase, Hfq, and GcvB, and how they affect gene expression in exponential and stationary-phase cells, will undoubtedly provide a wealth of fascinating information.

**Experimental procedures**

**Materials**

[γ-32P]ATP was purchased from PerkinElmer Life Sciences. T4 polynucleotide kinase, terminal transferase, Moloney murine leukemia virus–reverse transcriptase, RNase-free DNase I, and Taq2× Master Mix were products of New England Biolabs. DNA Clean & Concentrator™ was obtained from Zymo Research. QIAquick™ gel extraction kit was from Qiagen. TA cloning kit with PCR™ 2.1 vector and MEGAscript™ T7 transcription kit were from Thermo Fisher Scientific. SsoAdvanced™ Universal SYBR® Green Supermix and 0.45- and 0.2-μm nitrocellulose membranes were from Bio-Rad. Anti-mouse IgG HRP conjugate and calf intestinal alkaline phosphatase were obtained from Promega Corp. Thermo Sequence Cycle Sequencing kit was from Affymetrix Inc. SequaGel® urea gel system for denaturing urea-polyacrylamide gels was from National Diagnostics. Oligonucleotides, anti-FLAG M2 mAbs, and Amicon® ultracentrifugal units were obtained from Sigma. Nytran™ SPC nylon transfer membrane was from GE Healthcare. ExpressHyb™ hybridization solution was obtained from Clontech. All other chemicals were reagent grade.

**Bacterial strains and growth conditions**

Bacterial strains used in this study were derivatives of WT E. coli K-12 strain MG1655 (Seq rph+). The WT strain with chromosomal FLAG-tagged rbn was constructed in previous work (13). The WT strain with chromosomal FLAG-tagged hfq was a gift from Dr. Chaitanya Jain’s laboratory (University of Miami). Knockout strains of RppH, PAPI, Hfq, and lacZ were provided by Dr. Kenneth Rudd (University of Miami). The knockout strain lacking RNase G, the mutant strain RNase E (1–498), and the temperature-sensitive mutant strain RNase E− were from laboratory stocks. Null strains lacking the small noncoding RNAs (GlmZ, RyzB, RseX, GcvB, and Spf) and the rbn-Δ3’UTR strain were constructed by recombining (33) using the primers listed in Table S1. The triple knockout strain lacking GcvB, GlmZ, and RyzB (termed Δ3) and the double knockout strain lacking rbn and lacZ were constructed by transduction using phage P1 vir. The strains containing chromosomal FLAG-tagged rbn in the background of Δhfq, ΔgcvB, and Δ3 were also constructed by transduction using phage P1 vir.

Cells were grown in YT medium (Bacto-tryptone 8 g/liter, yeast extract 5 g/liter, sodium chloride 5 g/liter) at 30 or 37 °C. To inactivate RNase E, cultures grown at 30 °C were shifted to 42 °C and incubated for 20 min. Antibiotics, when present, were at the following concentrations: kanamycin, 25 μg/ml; ampicillin, 100 μg/ml; chloramphenicol, 17 μg/ml.

**β-Gal assay**

The rbn fragment −69 to +65 was amplified from chromosomal DNA of the WT strain using primers HC35 and HC36 in Table S1. PCR products were digested and ligated into the BamH I and Hind IIII restriction sites of the low-copy vector pLACZY1a (a gift from Dr. Chaitanya Jain) (34). The construct was introduced into E. coli strain MG1655 (Seq) rph+ Δrbn ΔlacZ.

Overnight cultures were diluted 1:200 into 200 ml of YT medium containing 100 μg/ml ampicillin. Cells were removed at different time points as indicated in Fig. 2D, followed by centrifuging at 4 °C. Cell pellets were resuspended in buffer Z (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, 50 mM β-mercaptoethanol). β-Gal activity was measured, as described previously (35), and was presented as Miller units by calculating the value 1000 × ((OD420 − 1.75 × OD550) / (t × V × OD600)), where t is time of the reactions in minutes, and V is volume of the cultures in milliliters.

**RNA decay**

Overnight cultures were diluted 1:200 into fresh YT medium and grown to exponential phase (A600 = 0.4) or stationary phase (A600 = 2.0) followed by treatment with 500 μg/ml rifampicin. Equal amounts of cells were removed for the zero-time sample after 75 s of rifampicin addition to allow complete inhibition of new transcription, and immediately mixed with ice-cold stop solution (95% ethanol, 5% phenol). Additional samples were removed at different time points as indicated in the figure legends. Changes in rbn mRNA levels were determined by quantitative RT-PCR (RT-qPCR) or RT-PCR.

**RNA extraction, RT-PCR, and RT-qPCR**

Total RNA was prepared using the hot phenol method described previously (36), and the RNA concentration was determined by UV spectroscopy. DNase I-treated RNA (1.2 μg)
was reverse-transcribed with the primers HC08 (rbn gene-specific primer), HC05 (16S RNA gene-specific primer), and HC12 (pnp gene-specific primer), using Moloney murine leukemia virus reverse transcriptase. The resulting cDNA was amplified by PCR using primers HC11 and HC02 to amplify rbn or using primers HC13 and HC14 to amplify pnp. PCR products were loaded on a 1.0% agarose gel and visualized using the Biodoc-it imaging system (UVP). The sequences of oligonucleotides are listed in Table S1.

To perform RT-qPCR analysis, 5 µl of 1:100 diluted cDNA was added to 20 µl of qPCR mixture together with 10 µl of SsoAdvanced™ universal SYBR® Green supermix and 0.5 µM gene-specific primers for rbn (HC09 and HC10) or 16S RNA (HC06 and HC07) as listed in Table S1. The amplification was carried out using MiniOpticon™ real-PCR system (Bio-Rad), and the data were analyzed using CFX Manager™ software (version 3.1).

5′-RACE assay

5′-RACE assay was performed as described previously (37). cDNA was synthesized from 2 µg of total RNA using an rbn-specific primer and was purified using DNA Clean & Concentrator™, followed by the addition of a poly(A) tail in a 20-µl reaction containing 2 µl of 10× reaction buffer, 2 µl of 10× CoCl₂ (2.5 mM), 4 µl of 1 mM ATP, and 20 units of terminal transferase. The reaction mixture was incubated for 15 min at 37 °C and was diluted to 500 µl using 1× Tris-EDTA buffer. The first round of amplification was performed using primers HC03, HC04, and HC02 (an outer rbn-specific primer), followed by the second round of amplification using primers HC03 and HC01 (an inner rbn-specific primer). PCR products were resolved in a 1.5% agarose gel and were recovered using QIAquick® gel-extraction kit. Purified DNA fragments were ligated with PCR™2.1 vector and were sequenced by Genewiz.

Northern blotting

Northern blot analysis was performed by fractionating 15 µg of RNA on 6% polyacrylamide, 7.5 M urea gels. RNA was then transferred onto nylon membranes by the semi-dry transfer method. The fixed membranes were first probed for 5S RNA using the probe 5′-ATGCTTGGCAGTTCTACTCTCGC-3′. Membranes were stripped and reprobed for GcvB using the probe 5′-CGGTGCTACATGATCTATGCCGAC-3′. Both probes were 5′-end-labeled using [γ-32P]ATP and T4 polynucleotide kinase. The blots were visualized using a STORM 840 phosphorimaging device. ImageQuant software was used to quantify the bands.

Measurement of RNase BN stability

Overnight cultures were diluted 1:200 into fresh YT medium and grown to exponential phase (A₆₀₀ = 0.4) or stationary phase (A₆₀₀ = 2.0), followed by the treatment with 400 µg/ml chloramphenicol. Equal amounts of cells were removed at different time points as indicated in the figure legends. Changes in RNase BN levels were determined by immunoblotting.

Immunoblotting

Total protein was prepared as described previously (38). Protein concentration was measured by the Bradford assay (39). Equal amounts of protein were separated by 10% SDS-polyacrylamide gels for RNase BN assay or by 15% SDS-polyacrylamide gels for Hfq assay and transferred onto nitrocellulose membranes. Membranes were treated with 3% nonfat milk and incubated with anti-FLAG M2 mAbs (1:1,000 dilution). RNases BN and Hfq were detected with HRP-conjugated anti-mouse IgG antibody and visualized with ECL solutions. Underexposed films were used for quantification using ImageQuant software.

Construction, expression, and purification of RNase E and Hfq

DNA fragments encoding Hfq and the N-terminal domain of RNase E (1–498) were amplified from chromosomal DNA of the WT strain using primers listed in Table S1. PCR products were digested and ligated into the Ndel and BamH I restriction sites of the pET15b expression vector. The constructs were introduced into E. coli strain BL21 (DE3) to overexpress Histagged Hfq and RNase E proteins.

Overnight cultures were diluted 1:100 into 500 ml of YT medium and grown to A₆₀₀ = 0.4 at 37 °C. Overexpression of Hfq and RNase E were induced for 3 h using 1 mM isopropyl-1-thio-β-D-galactopyranoside. Cultures were harvested by centrifugation, followed by protein purification as described in previous work (40) using buffer QE (20 mM Tris-Cl, pH 7.5, 500 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride). Eluted proteins in buffer QE containing 300 mM imidazole were filtered through Amicon® ultracentrifugals units to remove imidazole and to concentrate.

Substrate preparation

Templates for in vitro transcription of rbn fragment (1–165) and for GcvB were amplified by PCR from genomic DNA of the WT strain using primers listed in Table S1, followed by gel purification using the QIAquick® gel-extraction kit. In vitro transcription was performed according to instructions provided with the MEGASHortscript™ T7 transcription kit using 100 nM template. The two transcripts were purified by extracting the reaction mixture once with phenol/chloroform (1:1) and were precipitated overnight with ethanol at −20 °C. The 5′ terminus of purified rbn mRNA was labeled using [γ-32P]ATP and T4 polynucleotide kinase after treatment with calf intestinal alkaline phosphatase.

Gel-shift assay

GcvB and 5′-labeled rbn fragment (1–165) were denatured at 95 °C for 2 min, cooled on ice, followed by incubation at 37 °C for 10 min in 1× binding assay buffer (10 mM Tris, pH 7.0, 100 mM KCl, 10 mM MgCl₂). For all of the binding reactions (10 µl), 0.1 pmol of rbn was added to tubes containing different amounts of GcvB. Purified Hfq (10 pmol) was subsequently added, as needed. The mixture was incubated at 37 °C for 20 min. To determine whether the continued presence of Hfq was required for maintaining the complexes, 10 µg of proteinase K was used to remove Hfq. To examine interaction of rbn with GcvB in absence of Hfq, 1 pmol of rbn was incubated with increasing amounts of GcvB as indicated in figure legends. The reaction mixtures treated with proteinase K were extracted with phenol/chloroform (1:1) before mixing with 1 µl of native loading dye (50% glycerol, 0.01% bromphenol blue). Other reac-
tion mixtures were directly mixed with 1 μl of native loading dye. The mixtures were separated on a 5% native polyacrylamide gel. Electrophoresis was performed in 0.5× TBE at 200 V in a cold room for 2 h. Gels were subsequently analyzed using a STORM 840 phosphorimaging device.

**Primer extension analysis**

To identify cleavage sites of RNase E, 0.6 pmol of unlabeled rbn mRNA (1–165) was cleaved with purified RNase E at 37 °C using 1× reaction buffer (10 mM Tris-Cl, pH 7.0, 100 mM KCl, 10 mM MgCl2). Samples (60 μl) were removed at different time points as indicated in the figure legends, followed by extraction with phenol/chloroform (1:1) and precipitation with ethanol. Primer HC20 (listed in Table S1) was 5′-end-labeled using [γ-32P]ATP and T4 polynucleotide kinase. Labeled primer was used for both the sequencing reactions and primer-extension analysis. Cleavage products were mixed with labeled primer and dNTPs, heated to 80 °C for 5 min, and slowly cooled to 42 °C, followed by 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 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 instructions in the Thermo Sequence Cycle sequencing kit. Samples were resolved on 8% polyacrylamide, 7.5M urea gels in parallel with sequencing reaction samples, subsequently dried using the model 583 gel dryer (Bio-Rad), and analyzed using a STORM 840 phosphorimaging device.

**RNase E cleavage assay**

To identify the cleavage sites that are closer to 5′ end of rbn mRNA, 0.6 pmol of 5′-labeled rbn fragment (1–165) was cleaved with purified RNase E from RNase E cleavage, 5 pmol of GcvB and 60 pmol of Hfq protein were used. The reaction mixture lacking RNase E was incubated for 20 min at 37 °C, followed by addition of RNase E and additional incubation for 15 min. Reaction mixtures (60 μl) were purified by extraction with phenol/chloroform (1:1) and precipitation with ethanol. The cleavage products dissolved in diethyl pyrocarbonate/water were mixed with an equal volume of 2× loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol, and 0.05% xylene cyanol FF). The mixtures were resolved on an 8% polyacrylamide, 7.5M urea gel. After electrophoresis, gels were dried using the model 583 gel dryer (Bio-Rad) and analyzed using a STORM 840 phosphorimaging device.

**Prediction of interaction between sRNAs and rbn mRNA**

The list of Hfq-dependent small RNAs was from published data (41). The interaction between sRNAs and rbn mRNA was predicted by IntaRNA (20) using the sequences of sRNAs obtained from the BSRD database (42) and the complete sequence of rbn mRNA.

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