Initiation of decay of *Bacillus subtilis* *rpsO* mRNA by endoribonuclease RNase Y

SIYI YAO and DAVID H. BECHHOFER*

Department of Pharmacology and Systems Therapeutics, Box 1603, Mount Sinai School of Medicine of New York University, New York, NY 10029

Running title: RNase Y-mediated initiation of mRNA decay in *B. subtilis*

Key words: *Bacillus subtilis*, mRNA decay, *rpsO*, 3’ exonucleases, bacitracin-inducible transcription, RNase Y

* Corresponding author

Tel.: 212-241-5628

Fax: 212-996-7214

e-mail: david.bechhofer@mssm.edu
ABSTRACT

rpsO mRNA, a small monocistronic mRNA that encodes ribosomal protein S15, was used to study aspects of mRNA decay initiation in Bacillus subtilis. Decay of rpsO mRNA was analyzed in a panel of 3'-to-5' exoribonuclease mutants, using a 5'-proximal oligonucleotide probe and a series of oligonucleotide probes that were complementary to overlapping sequences starting at the 3' end. The results provided strong evidence that endonuclease cleavage in the body of the message, rather than degradation from the native 3' end, is the rate-determining step for mRNA decay. Subsequent to endonuclease cleavage, the upstream products were degraded by polynucleotide phosphorylase (PNPase) and the downstream products were degraded by the 5' exonuclease activity of RNase J1. rpsO mRNA half-life was unchanged in a strain that had decreased RNase J1 and no RNase J2, but was increased 2.3-fold in a strain with decreased RNase Y, a recently discovered ribonuclease of B. subtilis encoded by the ymdA gene. Accumulation of full-length rpsO mRNA and its decay intermediates was analyzed using a construct in which the rpsO transcription unit was under control of a bacitracin-inducible promoter. The results were consistent with RNase Y-mediated initiation of decay. This is the first report of a specific messenger RNA whose stability is determined by RNase Y.
INTRODUCTION

The rate of decay of a messenger RNA is important for determining the level of gene expression. Studies on the mechanism of mRNA decay in *Escherichia coli* have progressed based on a detailed knowledge of the ribonucleases involved in the process and the construction of ribonuclease mutant strains. A generally accepted model that applies to the turnover of many *E. coli* mRNAs has the 5'-end-dependent RNase E making the rate-determining endonuclease cleavage, which produces an upstream fragment that is subject to 3'-to-5' exonucleolytic decay by RNase II and a downstream fragment that is subject to further RNase E endonucleolytic cleavage (5, 24). Recent studies have suggested that, in some cases, a preliminary step in RNase E binding is conversion of the native triphosphate 5' end, which is a poor substrate for RNase E binding (22, 26, 40), to a monophosphate 5' end by a pyrophosphatase activity (4, 11). Degradation from the 3' end can also occur, and this is dependent on the 3' extending activity of poly(A) polymerase (16).

A similar level of understanding of the mechanism of mRNA decay has not been achieved for the model Gram-positive organism, *Bacillus subtilis*. Sequence homologues of some of the *E. coli* enzymes that play major roles in mRNA decay – e.g., RNase E, RNase II, poly(A) polymerase, – are not identifiable in the *B. subtilis* genome. Nevertheless, studies on a number of mRNAs, some of which are constitutively or inducibly stable, have suggested that mRNA decay in *B. subtilis* also initiates from the 5' end (9). *B. subtilis* PNPase, encoded by the *pnpA* gene, plays a major role in 3'-to-5' exonucleolytic degradation of decay intermediates (15, 32, 42). In addition to PNPase,
three other *B. subtilis* 3’-to-5’ exoribonucleases – RNase PH, RNase R, and YhaM – can participate in mRNA decay (32). Recently, the role of the RNase J enzymes (18) in *B. subtilis* mRNA turnover has become apparent. While the RNase J enzymes were initially purified on the basis of their endoribonuclease activity, it was shown subsequently that the essential RNase J1 also has 5’-to-3’ exoribonuclease activity (29), which is inhibited by a 5’-triphosphate end (14, 25). RNase J1 has been shown to be involved in decay and processing of a number of specific RNAs (2, 6, 14, 18, 45), and a transcriptome analysis demonstrated that hundreds of mRNAs had increased half-life in a strain that had reduced levels of RNase J1 and that was deleted for the non-essential RNase J2 (27).

Other endonucleases of *B. subtilis* that have been characterized to some extent are Bs-RNase III, RNase M5, RNase P, RNase Z, EndoA (8), and Mini-II (8, 37), none of which have been shown to be required for decay of an mRNA. Very recently, the product of the essential gene *ymdA* (20) has been renamed RNase Y by Stulke and colleagues, based on its association with other RNA processing enzymes and its apparent involvement in processing of the *gapA* operon mRNA (7). Putzer and colleagues showed that a strain that was depleted for RNase Y showed a significant increase in global mRNA half-life, suggesting that this enzyme plays a key role in mRNA turnover (38).

Previously, we used 5’-proximal oligonucleotide probes to analyze the steady-state decay pattern of a number of small monocistronic mRNAs, comparing the pattern detected by 5’-proximal probes in wild-type and *pnpA* strains (32). In each of seven cases, prominent decay intermediates were observed in the *pnpA* strain but not in the wild-type strain. One of these mRNAs was the *rpsO* mRNA, a 388-nucleotide (nt) mRNA that encodes ribosomal protein S15 (Fig. 1). The 3’ ends of the prominent *rpsO*
decay intermediates detected in the *pnpA* strain were mapped to the downstream side of predicted RNA structures. We suggested that the accumulation of these intermediates in the strains missing PNPase was the result of endonuclease cleavage downstream of the structure, followed by 3’-to-5’ exonuclease degradation up to the 3’ side of the structure.

In strains containing PNPase, decay intermediates were not readily detectable since PNPase could degrade past RNA structures. In the current study, we used *rpsO* mRNA to provide support for the endonucleolytic nature of decay initiation, and we show evidence that RNase Y is the decay-initiating endonuclease for *rpsO* mRNA.

**MATERIALS AND METHODS**

**Bacterial strains.** Triple and quadruple *B. subtilis* exoribonuclease mutant strains used in this study were derivatives of the parent strain BG1, which is *trpC2 thr-5* and which is designated “wild type.” Construction of exoribonuclease mutant strains has been described previously (32). Conditional endonuclease mutant strains used were: RNase J1 (2), RNase P (43), RNase Z (33), and RNase Y (23). These strains contained plasmid pMAP65 (34), which provided additional copies of *lac* repressor. Transformation of *B. subtilis* strains was as described (17).

**rpsO transcription under *p*bac control.** The *p*bac-*rpsO* construct was assembled as follows: A 90 base-pair fragment of the *lia* operon promoter, including the LiaR binding sites (28), was amplified by PCR, using an upstream oligonucleotide that included an MfeI restriction site. The *rpsO* transcriptional unit was amplified by PCR, using an upstream primer that contained a 7-nt sequence complementary to the beginning of the
downstream primer used for lia promoter region amplification, and a downstream primer that included a HindIII site after the rpsO transcription terminator. The two PCR amplicons were annealed and were amplified using the 5' lia promoter primer and the 3' rpsO primer. The resulting product was digested with MfeI and HindIII and cloned into the EcoRI and HindIII sites of plasmid pDR67-Pm, a derivative of the amyE integration plasmid pDR67 (21) in which the chloramphenicol resistance gene has been replaced by a phleomycin resistance gene (DHB, unpublished).

RNA analysis. RNA was isolated by hot phenol extraction from B. subtilis cultures grown in minimal medium containing Spizizen salts with 0.5% glucose, 0.1% casamino acids, 0.001% yeast extract, 50 µg/ml tryptophan and threonine, and 1 mM MgSO$_4$, as described (12). All strains were grown to late logarithmic growth stage (100 Klett units, using a #54 green filter), except for the quadruple 3' exoribonuclease mutant, which was grown to a density of 50 Klett units. For the experiment shown in Fig. 3D, strains were grown in 2X YT medium, containing 1% yeast extract, 2% tryptone, 1% NaCl, and the strain was grown to an OD$_{600}$ of 0.6. Expression of RNase J1 and RNase Y in the conditional mutant strains was induced with 1 mM IPTG. For induction of $\rho_{bac}$ transcription, bacitracin was added to a final concentration of 50 µg/ml when strains had grown to a density of 75 Klett units. Northern blot analysis of RNA separated on 6% (Figs. 2, 4, 5) or 9% (Fig. 3) denaturing polyacrylamide gels was done as previously described (19). 5'-end-labeled oligonucleotide probes were prepared using T4 polynucleotide kinase and [γ-$^{32}$P]ATP. To control for RNA loading, membranes were stripped and probed for 5S rRNA, as described (39).
**Data Analysis.** Quantitation of radioactivity in bands on Northern blots was done with a Storm 860 PhosphorImager instrument (Molecular Dynamics) or a Typhoon TRIO variable mode imager (GE Healthcare). *rpsO* mRNA half-life was determined by a linear regression analysis of percent RNA remaining vs. time. Half-life data was derived only from experiments in which the $R^2$ value was greater than 0.9. Comparison of wild-type and mutant RNA half-lives was used in a two-sample t test to derive P values. A P value <0.05 was considered significant.

**RESULTS**

**Half-life of *rpsO* mRNA in exonuclease mutants.** Experiments were done to determine whether the half-life of *rpsO* mRNA is affected by a deficiency of the known *B. subtilis* 3′-to-5′ exoribonucleases: PNPase, RNase R, RNase PH, and YhaM. The chemical half-life of the full-length *rpsO* mRNA was measured in strains that were deficient in three of the four exonucleases or all four exonucleases. The reasoning was as follows: If decay was initiated by endonucleolytic cleavage(s), then the half-life of the full-length mRNA should not be affected by the absence of any of the known exonucleolytic activities. The effect of the exonuclease deficiency would be primarily on the fate of decay intermediates that are produced by endonuclease cleavage.

Total RNA was isolated from a *B. subtilis* wild-type strain and from exonuclease mutant strains, at increasing times after rifampicin addition. Decay of *rpsO* mRNA was examined by Northern blot analysis, using a 5′-end-labeled oligonucleotide probe that
was complementary to the translation initiation region (nts 75-100) of the \textit{rpsO} message (5'-proximal probe, Fig. 1). The Northern blots are shown in Fig. 2 and the half-life data are presented in Table 1. The half-life of \textit{rpsO} mRNA in the wild-type strain was 3.2 minutes, and no prominent decay intermediates were detected (Fig. 2A). \textit{rpsO} mRNA half-life increased slightly (but not significantly) to 3.9 minutes in the strain that contained PNPase but was missing the other three exoribonucleases (Table 1). As in the wild-type strain, prominent decay intermediates were not detected (Fig. 2B). We hypothesize that, of the 3' exonucleases present in \textit{B. subtilis}, PNPase is the dominant activity in being able to degrade past strong secondary structure and eliminate decay intermediates (32). In the strain containing only RNase PH, many decay intermediates were detected, some of them in quantities that exceeded the amount of full-length mRNA (Fig. 2C; e.g., the “180-nt” RNA), and these decay intermediates were stable through the course of the experiment. The same pattern was obtained when a 5'-terminal probe that was complementary to nts 1-24 of \textit{rpsO} mRNA was used (Fig. 1; data not shown). Thus, the 5' end of these decay intermediates is likely to be at the transcription start site (TSS). Despite this massive accumulation of decay intermediates, the half-life of full-length \textit{rpsO} mRNA in the strain containing only RNase PH was 4.3 minutes, not significantly different from the half-life in the strain containing only PNPase (Table 1). The strain that contained only YhaM showed a similar pattern of decay intermediates as the strain containing only RNase PH (Fig. 2D), and the half-life was increased to 5.8 minutes (Table 1). In the strain containing only RNase R, a slightly different pattern of decay intermediates was detected (Fig. 2E), but the half-life of full-length \textit{rpsO} mRNA was also 5.8 minutes. Finally, in the strain that was deficient for all four of the known 3'-to-5'
exoribonucleases, the half-life of full-length *rpsO* mRNA was 4.9 minutes (Fig. 2F, Table 1). The observed half-lives did not correlate with the previously measured doubling times of the mutant strains (32). For example, the quadruple mutant has a doubling time that is 1.5-2-times longer than the triple mutants, yet the *rpsO* mRNA half-life was shorter in the quadruple mutant. These results indicated a minor role for 3’ exonuclease activity in determining the *rpsO* mRNA half-life. Rather, endonuclease cleavage was presumably the major determinant of mRNA half-life.

**Decay intermediates containing the 3’ end.** Endonuclease cleavage in the body of the *rpsO* message should generate upstream fragments containing the *rpsO* mRNA 5’ end (easily detected by the 5’-proximal probe in the PNPase-deficient mutants, as shown in Fig. 2) and downstream fragments containing the *rpsO* mRNA 3’ end. The stability of the downstream fragment would depend on its susceptibility to additional endonuclease cleavages or to the 5’-to-3’ exonuclease activity of RNase J1. Presumably, the presence of the transcription terminator structure at the 3’ end of the downstream fragment would protect against 3’-to-5’ exonucleolytic decay. We sought to detect such downstream fragments, using eleven overlapping oligonucleotide probes, each 36 nts in length, that were complementary to sequences starting from the *rpsO* transcription terminator past the midpoint of the coding sequence (CDS; Fig. 3A). These oligonucleotides were used in Northern blot analyses of RNA isolated from the wild-type strain (Fig. 3B). The blots were exposed for much longer times than those shown in Fig. 2. The results in Fig. 3B showed that, indeed, fragments that contained the *rpsO* mRNA 3’ end could be detected, although in much lower amounts than the full-length mRNA. Multiple species were detected, and the clearest groups of bands were labeled b-j, from largest to smallest (full-
length rpsO mRNA was labeled “a”). From the data, it was clear that the 5’ ends of the smallest fragments were closest to the 3’ end of rpsO mRNA, since they could only be detected by the 3’-proximal probes. The 5’ ends of the larger fragments were located increasingly further upstream, since they could be detected also by probes complementary to sequences further upstream in the CDS. If we assume that co-migrating bands observed with the different probes represent the same RNA fragments, then the pattern of these Northern blots demonstrated that all of the RNAs detected by these probes contained the 3’ end of rpsO mRNA. While there were insufficient amounts of these RNAs to map their 5’ ends precisely, the extent of these RNA fragments could be approximated, based on size markers run in parallel and assuming that the fragments share the same 3’ end. These are shown schematically in the lower part of Fig. 3A. Interestingly, the fragments appear to cluster in the 3’-proximal half of the transcript. We hypothesize that these RNAs result from one or more endonuclease cleavages downstream of the strong stem-loop near nt 180 and subsequent 5’-to-3’ processing. The fragments are detectable because they are protected by the transcription terminator structure, but, unlike the 5’-end-containing fragments (Fig. 2), they are unstable because they are susceptible to further endonucleolytic or 5’ exonucleolytic attack.

**Pattern of 3’-end-containing fragments in exoribonuclease mutants.** A considerable difference in the pattern of 5’-end-containing decay intermediates was observed between strains that did or did not contain PNPase (Fig. 2 panels A and B vs. C-F). According to our model, these decay intermediates arise by endonuclease cleavage in the body of the message, followed by 3’-to-5’ exonucleolytic decay up to the 3’ side of RNA structures, which block 3’ exonucleases other than PNPase (32). On the other
hand, we predicted that the pattern of 3'-end-containing decay fragments (Fig. 3B), which are the downstream fragments resulting from endonuclease cleavage, should not be affected significantly by the type of 3’ exonuclease activity present in the cell. This was tested directly by Northern blot analysis of 3'-end-containing fragments in the various triple exonuclease mutant strains, using the 3’-terminal probe 1 (Fig. 3C). The results showed that the pattern of 3'-end-containing fragments in the wild-type and ribonuclease mutant strains, although not identical, was similar in terms of sizes and amounts of RNA fragments.

**Level of 3'-end-containing fragments in RNase J mutant strains.** Although 3'-end-containing fragments were detectable in the wild-type strain, they were in relatively low abundance. We tested whether RNase J1 or RNase J2 was responsible for degradation of these fragments by repeating the Northern blot analysis using probe 1 on RNA isolated from RNase J mutant strains. The RNase J1 mutant strain has *rnjA* expression under control of an IPTG-inducible promoter. When grown in the absence of IPTG, the RNase J1 mutant strain contains a severely decreased level of enzyme, while growth in the presence of IPTG results in about a five-fold decreased level of enzyme (10). As can be seen in Fig. 3D, the steady-state pattern of small fragments showed somewhat increased intensity in the RNase J1 mutant strain grown with IPTG but sharply increased intensity when grown without IPTG. The deletion of RNase J2 gene did not show a clear effect on the decay intermediate pattern. (In this experiment, the amount of full-length RNA detected in the RNase J2 mutant lane was about 20% greater than in the wild-type lane. Hence, the lower bands in the RNase J2 mutant lane are more visible than in the wild-type lane, but the patterns are the same.) We conclude that RNase J1 is
rpsO mRNA half-life in endonuclease mutant strains. At the time this study of rpsO mRNA decay was begun, RNase J1 was the only endonuclease of B. subtilis known to be involved in initiation of mRNA decay. We thus thought that RNase J1 could be responsible for the endonucleolytic cleavage(s) that were suggested by the analysis of rpsO mRNA decay intermediates. Northern blot analysis was used to measure rpsO mRNA half-life in the RNase J1 conditional mutant strain, grown in the presence of 1 mM IPTG. The decrease in RNase J1 levels in the conditional mutant strain, even when IPTG is present, is sufficient to detect changes in the half-life of RNAs whose decay is RNase J1-dependent (10, 13, 44). We found, however, that the half-life of rpsO mRNA in the RNase J1 mutant was 3.9 min (data not shown), not significantly different from the 3.2 min half-life in the wild type. The half-life in an RNase J1 mutant strain in which the RNase J2 gene was deleted was similar (3.8 min). More recent reports have identified RNase Y as potentially a major player in RNA decay and processing in B. subtilis (see Discussion). We therefore measured the half-life of rpsO mRNA in the RNase Y conditional mutant, grown in the presence of 1 mM IPTG. The growth rate of the RNase Y mutant strain in these conditions was only slightly slower than the wild type (data not shown). Northern blot analysis of rpsO mRNA decay in the wild-type and RNase Y mutant strains after addition of rifampicin showed a 2.3-fold increase in mRNA half-life (Fig. 4).

A system to follow appearance of decay intermediates over time. The prominent rpsO mRNA decay intermediates observed at steady state in the PNPase-
deficient strains (Fig. 2) suggested that we could use an inducible system to follow accumulation of these fragments over time, to confirm the involvement of a particular endonuclease in initiating decay. Several problems were encountered when we did time-course analyses using the conventional IPTG-inducible or xylose-inducible promoters.

First, since the rpsO 5'-end-containing decay intermediates were extremely stable (Fig. 2), we needed an inducible transcription system that was not “leaky,” as ongoing low-level transcription in the absence of inducer would lead to steady-state accumulation of RNA fragments. The IPTG- and xylose-inducible systems, which are negatively regulated by repressors, were somewhat leaky (data not shown). Second, an examination of appearance of decay intermediates requires an induction system that scales up to full induction over a reasonably extended period of time. We found that full induction occurred over an extremely short time period when IPTG- and xylose-inducible systems were used (data not shown). Third, regulation of the commonly-used inducible promoters depends on the presence of an operator sequence located downstream of the TSS. This means that the induced transcript necessarily contains the cognate operator sequence, which gives a very different 5'-proximal sequence from the native transcript. This may be problematic for studying mRNA decay that could be 5'-end dependent.

To avoid some of these issues, we created a new inducible system that relies on the bacitracin-inducible promoter of the lia operon (28). This promoter is induced positively by subinhibitory concentrations of bacitracin, and we call it the “p_{bac}” promoter. A 90 base-pair fragment encompasses the promoter and the upstream regulatory region at which the transcriptional activator, LiaR, binds when bacitracin is present (28). A construct was integrated at the amyE locus that had the p_{bac} promoter
fragment located such that transcription started at the \( rpsO \) TSS to give “\( p_{bac}-rpsO \) mRNA.” To differentiate between native \( rpsO \) mRNA and \( p_{bac}-rpsO \) mRNA, nts 8-16 of the \( rpsO \) sequence (UAAAACCAU) were changed to the complementary sequence. 

\( B.\) subtilis \( rpsO \) mRNA begins with a leader region (Fig. 1), which includes a predicted pseudoknot structure (41) that is thought to be involved in translational autoregulation (35, 36). However, the pseudoknot structure begins at nt 30, and we assumed that changing nts 8-16 would not affect decay characteristics of the message. In these experiments, we used a bacitracin concentration of 50 µg/ml (the equivalent of 3.5 U/ml), which gives full induction but does not significantly affect bacterial growth (31).

A Northern blot analysis of induction kinetics of \( p_{bac}-rpsO \) mRNA in the \( pnpA \) strain is shown in Fig. 5A. The probe was directed to the 5’ end of \( p_{bac}-rpsO \) mRNA. Before addition of bacitracin (lane B) no signal is detected, demonstrating that the probe does not detect native \( rpsO \) mRNA. The \( p_{bac}-rpsO \) mRNA is detected faintly at 1 minute and increases over time, with almost full induction at around 15 minutes (cf. Fig. 5B). At this time, the prominent 180-nt decay intermediate is visible, suggesting that the nature of the promoter and the change in 5’-proximal sequence does not affect mRNA processing.

**Induction of \( p_{bac}-rpsO \) mRNA in endonuclease mutants.** A time course of accumulation of full-length \( p_{bac}-rpsO \) mRNA and decay intermediates was performed in several endonuclease mutant backgrounds. No difference was seen between the single mutant \( pnpA \) strain and the \( pnpA \) strain that had decreased levels of RNase J1 and no RNase J2 (Fig. 5B). This correlated with the similar half-life of \( rpsO \) in wild-type and RNase J1 mutant strains, as described above. Similarly, the bacitracin-induced time course was unaffected in strains with reduced levels of RNase P and RNase Z (data not
shown). However, the results with the RNase Y mutant strain were informative. At the earliest time point (0.5 min), p_{bac}-rpsO mRNA was virtually undetectable in the \textit{pnpA} strain, but was clearly present in the double mutant strain with reduced RNase Y (Fig. 5C). Over time, the amount of 180-nt decay intermediate, relative to full-length p_{bac}-rpsO mRNA, accumulated to a much higher degree in the \textit{pnpA} strain than in the double mutant strain with reduced RNase Y (Fig. 5D). These results are consistent with initiation of decay by RNase Y cleavage. One other prominent RNA, about 270 nts, was detected, but quantitative analysis of this RNA showed that it did not accumulate relative to the full-length RNA (Fig. 5D).

**DISCUSSION**

The analysis of \textit{rpsO} mRNA half-life in 3’ exonuclease mutants (Table 1, Fig. 2) indicated a minor role of 3’-to-5’ exoribonucleases in determining mRNA half-life. We observed slight but not statistically significant increases in \textit{rpsO} mRNA half-life in strains containing only PNPase or only RNase PH, and less than two-fold increases in \textit{rpsO} mRNA half-life in strains containing only YhaM or only RNase R, or none of the known 3’ exonucleases. These results suggested some effect of 3’ exonucleases on the half-life of full-length \textit{rpsO} mRNA, but endonuclease cleavage was likely more important for initiation of decay. We have no good explanation at present for the larger increase in \textit{rpsO} mRNA half-life in the strains containing only YhaM or RNase R, especially since this increase was larger than in the strain containing none of the four
known exoribonucleases (Table 1). Perhaps particular perturbations in the exoribonuclease complement of the cell indirectly affects endoribonuclease activity; recent evidence for a putative *B. subtilis* degradosome complex that includes PNPase, RNase J1, and RNase Y (7) may be indicative of other interactions between ribonucleases.

Endonuclease cleavage in the *rpsO* mRNA decay pathway was also inferred from the detection of multiple RNA fragments of increasing size but all containing the 3’ end (Fig. 3B). We found that this pattern was similar in the wild-type and exoribonuclease mutant strains examined (Fig. 3C), despite the enormous differences between these strains in the pattern detected with the 5’-proximal probe (Fig. 2). The contrast between the complete lack of 5’-proximal decay intermediates in the strain containing only PNPase (Fig. 2B) and the presence of 3’-proximal decay intermediates in this same strain (Fig. 3C, lane A) is particularly striking, and is consistent with decay initiation by endonuclease cleavage. The 3’-terminal fragments may be a direct product of endonuclease cleavage, or they may be secondary products of RNase J1 5’ exonuclease activity that proceeds from the 5’ end(s) generated by endonuclease cleavage. The low level of 3’-end-containing fragments in the wild-type strain is likely because RNase J1 is capable of degrading through secondary structure in the 5’-to-3’ direction (14). Indeed, depleting the cell severely (-IPTG) or moderately (+IPTG) of RNase J1 resulted in increased intensity of the 3’-end-containing decay intermediates (Fig. 3D).

We found in assays of mRNA half-life and of accumulation of full-length RNA and decay intermediates that RNase J1 was not responsible for determining the stability of *rpsO* mRNA. Although RNase Y, the product of the *ymdA* gene, was suspected long
ago of being a ribonuclease (1, 9), only in the last year has data been published on the role of RNase Y in RNA processing. Stulke and colleagues found evidence that cleavage of gapA operon mRNA at a particular site (30) was due to RNase Y (7). Putzer and colleagues demonstrated that cleavage of the yitJ riboswitch RNA, as well as other S-adenosylmethionine-dependent riboswitches, could be attributed to RNase Y (38). They found further that depletion of RNase Y resulted in an increase in global mRNA half-life. Thus, we turned our attention to RNase Y, and showed, for the first time, an effect of RNase Y on decay of a specific B. subtilis messenger RNA. We observed more than a two-fold increase in rpsO mRNA half-life in the RNase Y conditional mutant (Fig. 4). This suggests a strong dependence on RNase Y for initiation of decay, since we can assume that the RNase Y conditional mutant grown in 1 mM IPTG retains a significant level of the enzyme. We have not proven that RNase Y acts directly on rpsO mRNA, which will require tests in vitro, and it is possible that a deficiency in RNase Y results in indirect effects on initiation of mRNA decay. Nevertheless, the simplest interpretation of our results is that RNase Y cleaves rpsO mRNA, and the discussion below makes this assumption.

The pbac system was useful in demonstrating a faster accumulation of full-length rpsO mRNA in the RNase Y mutant strain (Fig. 5C, zero time point), presumably because initiation of its decay was slower due to the lower level of RNase Y. We hypothesize that the 180-nt RNA arises due to endonuclease cleavage followed by 3’ exonuclease activity up to the strong stem-loop structure that ends at nt 172. Thus, if RNase Y cleavage is required to generate this RNA, its accumulation should be slower in the RNase Y mutant, and this was indeed the case (Fig. 5C, 5D). Other bands, in addition
to the full-length and 180-nt band, were detected (Fig. 5C). Some of these are faint and are present throughout the time course, and these likely represent non-specific hybridization. We speculate that the band at around 270 nts may be a prematurely terminated transcription product or a processing product of a different ribonuclease on full-length RNA.

From the current analysis of *rpsO* mRNA decay intermediates, it was not clear if RNase Y cleaves once or several times in the body of the message. Even a single endonuclease cleavage could give rise to numerous decay intermediates by subsequent 3'-to-5' exonuclease and 5'-to-3' exonuclease activities and hindrance of these activities by RNA structure. In any event, the results to date allow building of a preliminary model for the complete turnover of *rpsO* mRNA, which begins with endonuclease cleavage by RNase Y and is completed by the 3' exonuclease activity of PNPase on upstream products and the 5' exonuclease activity of RNase J1 on downstream products (Fig. 6).

As RNase Y is essential, we expect that RNase Y will catalyze decay-initiating cleavage of many *B. subtilis* mRNAs.

Unlike RNase J1, which has robust endonuclease activity on 5' triphosphorylated substrates (14, 25), RNase Y endonuclease activity is sensitive to the nature of the 5' end, with significantly higher *in vitro* activity on RNA with a 5' monophosphorylated end (38). If this observation is true also of RNase Y activity *in vivo*, this will have major consequences for models of RNase Y-dependent initiation of decay. The data in Fig. 2 show that decay intermediates detected in the PNPase-deficient strains were extremely stable, and did not decrease in intensity throughout the duration of the experiment. This suggests that degradation by RNase J1 5' exonucleolytic activity from the native 5' end...
was not occurring, even though the data from Fig. 3D indicate that this same 5’ exonuclease activity was degrading the 3’-end-containing fragments. This suggests that the 5’ triphosphate group of the initial \textit{rpsO} transcription product is not being removed, making 5’-end-containing decay intermediates resistant to RNase J1 5’ exonucleolytic decay. However, if this is the case, and RNase Y is sensitive to 5’-triphosphate end, then it is hard to understand how internal cleavage by RNase Y to give a relatively short half-life (3.2 minutes) is being achieved. Much work is needed to understand the basis of endonucleolytic cleavage by RNase J1 and RNase Y, and to learn why particular messages are subject to one or the other, or perhaps both, activities.

\section*{Acknowledgements}

This work was supported by Public Health Service grant GM-48804 from the National Institutes of Health to D.H.B.
Table 1. Ribonuclease mutants and *rpsO* half-lives.

<table>
<thead>
<tr>
<th>Mutant type</th>
<th>RNase(s) present&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Exoribonuclease genotype&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Half-life&lt;sup&gt;c&lt;/sup&gt; (Mean ± SD)</th>
<th>P value&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>PNPase</td>
<td>n/a</td>
<td>3.24 ± 0.47</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>RNase PH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>YhaM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RNase R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triple</td>
<td>PNPase</td>
<td>*rnr:*Tc <em>rph</em>ΩSp *yhaM:*Pm</td>
<td>3.94 ± 0.39</td>
<td>0.063</td>
</tr>
<tr>
<td></td>
<td>RNase PH</td>
<td>*pnpA:*Cm *rnr:*Tc *yhaM:*Pm</td>
<td>4.30 ± 0.73</td>
<td>0.142</td>
</tr>
<tr>
<td></td>
<td>YhaM</td>
<td>*pnpA:*Km *rnr:*Tc <em>rph</em>ΩSp</td>
<td>5.84 ± 0.82</td>
<td>0.035</td>
</tr>
<tr>
<td></td>
<td>RNase R</td>
<td>*pnpA:*Km <em>rph</em>ΩSp *yhaM:*Pm</td>
<td>5.76 ± 0.65</td>
<td>0.025</td>
</tr>
<tr>
<td>Quadruple</td>
<td>none</td>
<td>*pnpA:*Cm *rnr:*Tc <em>rph</em>ΩSp *yhaM:*Pm</td>
<td>4.85 ± 0.15</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

<sup>a</sup> Of the four known 3’-to-5’ exoribonucleases.

<sup>b</sup> Resistance markers were: Cm, chloramphenicol; Km, kanamycin; Pm, phleomycin; Sp, spectinomycin; Tc, tetracycline.

<sup>c</sup> Data are the results of three experiments.

<sup>d</sup> P values: Half-lives for the mutant strains were compared to wild type.
Fig. 1. Schematic diagram of \( rpsO \) transcript, showing location of Shine-Dalgarno sequence (vertical rectangle), start and stop codons, four predicted stem-loop structures which appear to block 3’ exoribonuclease processivity (32), and 3’ transcription terminator (3’T). The relative predicted strength of the stem-loop structures is indicated roughly by the size of the stems. Also indicated is the location of complementary probes used in Northern blot experiments to detect 5’-end-containing decay intermediates.

Fig. 2. Northern blot analysis of \( rpsO \) mRNA decay in 3’-to-5’ exoribonuclease mutants. The genotype with respect to the four known 3’ exoribonucleases is shown below each panel. Above each lane is the time (min) after rifampicin addition. The probe was a 5’-end-labeled oligonucleotide complementary to the \( rpsO \) translation initiation region (nts 75-100; Fig. 1). Full-length \( rpsO \) mRNA is indicated by the arrow at the left of each panel. The prominent 180-nt decay intermediate is indicated at right. Quantity of total RNA in each lane was corrected according to the amount of RNA detected by a 5S rRNA-specific oligonucleotide probe, shown at the bottom of each panel.

Fig. 3. Detection of 3’-end-containing mRNA decay fragments. (A) Linear diagram of \( rpsO \) transcript. The box representing the CDS in Fig. 3A corresponds to the interval between the AUG start and UAA stop codons shown in Fig. 1. The region of complementarity for each of 11 overlapping 3’-proximal probes (numbered 1-11) used in the Northern blots of panel B is shown. The approximate extent of 3’-end-containing
mRNA decay fragments is shown schematically below the probes, using the lettering indicated in panel B. “a” is full-length rpsO mRNA, for which the stem-loop that ends at nt 172 (see Fig. 1) and that gives rise to the “180-nt” decay intermediate is shown for reference. The diagrams of RNAs b - j are meant to represent not single RNAs but groups of RNAs with nearby 5’ endpoints. (B) Northern blot analyses of 3’-end-containing rpsO decay fragments in the wild-type strain, with probe number indicated below each blot. Groups of fragments are labeled b - j, to the right of each lane containing total RNA. The marker lane (M), on the left side of the blots, contained 5’-end-labeled fragments of a TaqI digest of plasmid pSE420 (3). Values to the left indicate molecular sizes in nucleotides. (C) Northern blot analysis of 3’-end-containing fragments in triple ribonuclease mutants, using probe 1. Wild type (wt) contained all four known 3’-to-5’ exoribonucleases. The genotype with respect to the four known 3’ exoribonucleases is shown at bottom right of the blot for lanes A-D. Marker (lane M) is as described for panel B. (D) Northern blot analysis of 3’-end-containing fragments in RNase J1 and J2 mutant strains. RNase J1 conditional mutant was grown in the presence or absence of IPTG, as indicated. Marker (lane M) is as described for panel B.

Fig. 4. Northern blot analysis of rpsO mRNA decay in wild-type and RNase Y mutant strains. The probe was the 5’-proximal probe (Fig. 1). Above each lane is the time (min) after rifampicin addition. Measured half-life (avg. of three experiments) ± S.D. is shown beneath each blot. Marker lane at left is as described for Fig. 3B.
**Fig. 5.** Time course of $p_{bac}$-$rpsO$ mRNA accumulation. (A) Induction of $p_{bac}$-$rpsO$ transcription. Migration of full-length (FL) $p_{bac}$-$rpsO$ and prominent decay intermediate (180 nts) is indicated at right. Prominent decay intermediate of ~180 nts is indicated at right. Time (min) after addition of bacitracin is at the top of each lane. The “zero” time point in parts A-C is actually the interval between addition of bacitracin and removal and processing of the first aliquot, which is about 30 seconds. Lane B is RNA isolated before addition of bacitracin. Marker (lane M) is as described for Fig. 3B. (B) Accumulation of decay intermediates in the $pnpA$ strain and in the $pnpA$ strain with a reduced level of RNase J1 and no RNase J2. (C) Appearance of decay intermediates in the $pnpA$ strain and the $pnpA$ strain with reduced RNase Y. (D) Ratio of the 180-nt decay intermediate (solid lines) and 270-nt decay intermediate (dashed lines) in the $pnpA$ strain (diamonds) and the $pnpA Pspac-rny$ double mutant strain (squares). Error bars are shown only for the 180-nt decay intermediate. Data points are identified on the right by ribonuclease genotype and fragment size in parentheses.

**Fig. 6.** Model for pathway of $rpsO$ mRNA decay. Only a single RNase Y endonuclease cleavage is shown, but there may be additional cleavage sites.
References


