5′ End-independent RNase J1 Endonuclease Cleavage of Bacillus subtilis Model RNA*

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Background: Endonuclease cleavage initiates messenger RNA decay in bacteria.

Result: Cleavage of a model RNA by the endonuclease RNase J1 is unaffected by strong secondary structure at the 5′ end.

Conclusion: RNase J1-initiated RNA decay occurs by endonuclease cleavage independent of the 5′ end.

Significance: Although mRNA stability is generally 5′ end-dependent, mRNAs may contain sites that circumvent this dependence.

Bacillus subtilis trp leader RNA is a small (140-nucleotide) RNA that results from attenuation of trp operon transcription upon binding of the regulatory TRAP complex. Previously, endonucleolytic cleavage by ribonuclease RNase J1 in a 3′-proximal, single-stranded region was shown to be critical for initiation of trp leader RNA decay. RNase J1 is a dual-specificity enzyme, with both 5′ exonucleolytic and endonucleolytic activities. Here, we provide in vivo and in vitro evidence that RNase J1 accesses its internal target site on trp leader RNA in a 5′ end-independent manner. This has important implications for the role of RNase J1 in RNA decay. We also tested the involvement in trp leader RNA decay of the more recently discovered endonuclease RNase Y. Half-lives of several trp leader RNA constructs, which were designed to probe pathways of endonucleolytic versus exonucleolytic decay, were measured in an RNase Y-deficient mutant. Remarkably, the half-lives of these constructs were indistinguishable from their half-lives in an RNase J1-deficient mutant. These results suggest that lowering RNase Y concentration may affect RNA decay indirectly via an effect on RNase J1, which is thought to exist with RNase Y in a degradosome complex. To generalize our findings with trp leader RNA to other RNAs, we show that the mechanism of trp leader RNA decay is not dependent on TRAP binding.

One of the small RNAs that we have studied intensively is trp leader RNA (see Fig. 1A), a 140-nucleotide (nt)2 RNA that is transcribed from the constitutive trp promoter. When tryptophan synthesis is needed, transcription of the trp operon results in formation of an antiterminator structure which allows transcription of the full-length trp operon. When tryptophan synthesis is not needed, excess tryptophan binds to a regulatory protein named TRAP (trp RNA-binding attenuation protein), which exists as an 11-mer complex. The tryptophan-activated TRAP complex binds to 11 trinucleotide repeat sequences located between nt 36–91 of the trp leader region RNA. TRAP binding masks nucleotides that are part of the antiterminator structure, allowing formation of the trp leader terminator structure and transcription termination at nt 140 (2, 3). As shown in Fig. 1A, trp leader RNA has a 5′-terminal stem-loop structure (5′-SL; nt 2–32) that is important for TRAP binding (4), an internal region that is the TRAP binding site (TBS; nt 36–91), and a 3′-terminal transcription terminator structure (3′-TT; nt 108–133). Thus, except for a short single-stranded sequence between nt 92–107, virtually all of the trp leader RNA is highly structured or bound by protein, a situation that is usually associated with RNA stability. However, trp leader RNA must be degraded rapidly in order to release bound TRAP for ongoing transcription attenuation. Inhibiting trp leader RNA decay results in inappropriate transcription of the full-length trp operon, even when tryptophan is plentiful (5).

We have shown that the relatively short 2.5-min half-life of trp leader RNA is due to initiation of decay by RNase J1 endonucleolytic cleavage at around nt 101 (6–8). RNase J1 of B. subtilis is an unusual enzyme in that it specifies two ribonuclease activities: an endonuclease activity with cleavage specificity similar to that of Escherichia coli RNase E (9–11) and a 5′-to-3′-exonuclease activity that is the first such activity to be described in prokaryotes (12). The 5′-exonuclease activity is sensitive to the RNA 5′ phosphorylation state, being active on RNAs with a 5′-monophosphate end and virtually inactive on RNAs with a 5′-triphosphate end (7, 10). RNase J1 is an essen-

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tial enzyme, whereas a related enzyme, RNase J2, which specifies a similar endonuclease activity but not 5′-exonuclease activity (13), is not essential (9). RNases J1 and J2 have been shown to exist primarily in a heterotetrameric complex (13). The half-life of a number of B. subtilis RNAs, including trp leader RNA, are known to depend on RNase J enzymes, as shown in strains in which RNase J1 is expressed from an IPTG-inducible promoter and is present at lower than wild-type concentrations, and/or the RNase J2 gene is deleted (14).

Previous studies (5–8) on trp leader RNA have led to the following model for its turnover: initiation of decay occurs by RNase J1 cleavage at around nt 101, which is located in an AU-rich sequence AUUAUGULUAUU (nt 101 is underlined). Endonucleolytic cleavage at nt 101 leaves an upstream fragment with a susceptible 3′ end that is attacked primarily by the 3′-to-5′-exonuclease, polynucleotide phosphorylase (PNPase). Although at least three other 3′-exonucleases are present in the cell (15), only PNPase is capable of degrading past TRAP-bound RNA, which results in the timely release of TRAP that is then available to bind nascent trp transcripts. The ~40-nt downstream fragment of RNase J1 cleavage is degraded by the 5′-exonuclease activity of RNase J1 itself. The acute dependence of trp leader RNA half-life on RNase J1 endonucleolytic cleavage is demonstrated by the effect of replacing AU sequences around nt 101 with a GC sequence GCGGCCGC (yielding “NotI RNA” after the NotI restriction endonuclease site that this sequence constitutes in DNA; see Fig. 2A). This sequence change results in a 4-fold increase in trp leader RNA half-life from 2.5 min to 10.3 min.

The possible involvement of RNase J1 5′-exonuclease activity in trp leader RNA decay was suggested by the fact that addition of an A7 sequence to the 5′ end of trp leader RNA reduced its half-life from 2.5 to 0.9 min (8). Similarly, addition of A7 to the 5′ end of NotI RNA reduced its half-life from 10.3 to 3.2 min. These and other results provided evidence that the presence of an unstructured 5′ sequence could enable decay via RNase J1 exonuclease activity from the native 5′ end, presumably only after conversion of the 5′-triphosphate end to a 5′-monophosphate end by an RNA pyrophosphohydrolase activity (16, 17). However, it was not determined whether the native 5′-SL structure of trp leader RNA inhibits RNase J1 5′-exonuclease activity. Most surprisingly, addition of a strong stem structure (Fig. 1B) at the 5′ end of the construct with the added A7 sequence (yielding “5′-SSS RNA”) only increased the half-life from 0.9 min to the wild-type 2.3 min. This latter result is striking. Although it was expected that placement of a 5′-terminal strong stem structure would negate the destabilizing effect of the added A7 tail, it was thought that the presence of such a structure would also negatively affect the efficiency of RNase J1 endonuclease cleavage. The wild-type half-life of 5′-SSS RNA suggested that access by RNase J1 to its internal target site occurs independently of the 5′ end.

In this report, we provide more evidence for “direct access” of RNase J1 to the nt 101 cleavage site, and we explore the involvement of RNase J1 5′-exonuclease activity in trp leader RNA decay. The role that the more recently discovered RNase Y (18, 19), which is thought to play an even larger role than RNase J1 in bulk mRNA turnover (19), plays in trp leader RNA was also assessed. In addition, derivatives of trp leader RNA were constructed to show that the mechanism of trp leader RNA decay was not dependent on its unique characteristics, most notably TRAP binding, which suggests that trp leader RNA can serve as a model for general RNA decay mechanisms. Finally, we revisit an unusual block to PNPase activity.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**—BG626 was the host strain for plasmids carrying the wild-type or mutant trp leader constructs. BG626 is trpC2 thr-5 and is deleted for the endogenous trp leader sequence, as described previously (6). For experiments with reduced RNase J1 or RNase Y expression from the IPTG-inducible pBAD promoter, BG626 was transformed to erythromycin resistance using chromosomal DNA from the RNase J1- (20) or RNase Y-conditional mutant strains (19). The conditional mutant strains also contained plasmid pMAP65, which carries extra copies of the lacI gene for stronger repression (21). For determination of trp leader RNA half-life in the absence of RNase J2, an mtnB deletion strain (20) was transformed with pGD5 (see below). The host for cloning of mutant trp leader constructs was E. coli DH5α (22).

**Plasmids**—trp leader RNA mutants were carried on derivatives of plasmid pGD5 (6), which contains the wild-type trp leader sequence and the mtnB gene encoding TRAP, and were constructed as described (8). The selective marker on the initial set of plasmids was tetracycline resistance. For experiments in the pnpA strain, a tetracycline-resistance marker could not be used since the strain is hyper-sensitive to tetracycline (23). For these experiments, the tetracycline-resistance marker was replaced by a chloramphenicol-resistance marker.

**Purification of RNase J1 and H76A Mutant Protein**—Over-expression and purification of RNase J1, as well as the H76A mutant protein, were as described (24).

**Preparation of RNA Substrates**—trp leader RNA was synthesized using T7 RNA polymerase transcription (Ambion T7 MAXIscript kit) of a PCR fragment that contained the trp
leader sequence, with a T7 promoter included in the upstream primer. The transcription product was purified as described previously (7). 5' end-labeled trp leader RNA was added to 1 mM for induction of essential ribonuclease gene expression. Northern blot analysis was performed as described (5). To control for RNA loading in Northern blot analyses, membranes were stripped and probed for 5S rRNA as described (26). 5' end-labeled oligonucleotides were used as probes. The 5' probe was complementary to nt 1–20. The trp-specific 3' probe was complementary to nt 99–125 of trp leader RNA. The rpsO-specific 3' probe was complementary to nt 99–125 of trp:rpsO-TT RNA. For all half-life measurements $R^2$ values were greater than 0.9. Comparison of the half-lives between two trp leader RNAs was used in a two-sample $t$ test to derive $p$ values. A $p$ value $< 0.05$ was considered significant.

RESULTS AND DISCUSSION

Half-lives of trp Leader RNA and Derivatives in Wild-type and RNase J1 Mutant Strains—Ribonuclease wild-type and RNase J1 mutant strains were constructed that were deleted for the native trp leader RNA locus and carried wild-type or mutant trp leader RNA on a plasmid. The plasmids also contained the mtrB gene encoding TRAP, to provide sufficient TRAP for the wild-type strain. In the RNase J1 mutant strains, expression of chromosomal RNase J1 was under control of the IPTG-inducible $p_{spa}$ promoter. In the presence of 1 mM IPTG, expression of RNase J1 is ~5-fold lower than in the wild-type (27). The same slope was measured for growth curves of the RNase J1 mutant strain grown with 1 mM IPTG and the wild-type strain (data not shown), eliminating the possibility that changes in half-life were due to altered growth of the mutant strain.

In addition to the wild-type trp leader RNA, NotI RNA, and 5'-SSS RNA constructed previously (Fig. 2A), we made a derivative of 5'-SSS RNA that contained the NotI site at nt 101 (yielding "5'-SSS+NotI RNA"). The half-lives of these RNAs in the wild-type strain are shown in the top row of values in Fig. 2A. As observed previously (6), mutating the AU-rich sequence around nt 101 in NotI RNA dramatically increased the half-life. An in vitro assay with purified RNase J1 was used to show that the change from an AU-rich to GC-rich sequence completely eliminated cleavage of trp leader RNA (Fig. 2B). As mentioned in the Introduction, the half-life of 5'-SSS RNA was the same as trp leader RNA despite the presence of the 5'-terminal struct-
The half-life of 5′-SSS + NotI RNA (11.7 min) was only slightly higher than NotI RNA itself (10.3 min; p value = 0.02). The second row of values in Fig. 2A shows the half-lives for these RNAs in the RNase J1 mutant strain. We observed previously that lowering cellular RNase J1 concentration resulted in a 2-fold increase in half-life (to 5.4 min) for wild-type trp leader RNA (8). Here, we found this to be the case also for 5′-SSS RNA (5.6 min), again suggesting that placement of the strongly structured stem-loop at the 5′ end had no effect on RNA decay. NotI RNA half-life was slightly, although significantly, different in the RNase J1 mutant and wild-type strains (9.1 min and 10.3 min, respectively; p value = 0.01). Interestingly, 5′-SSS + NotI RNA half-life was 50% longer (17.6 min) in the RNase J1 strain than the same RNA in the wild-type strain (11.7 min).

Our hypothesis to explain these findings was as follows: the dominant pathway for initiation of trp leader RNA decay is RNase J1 cleavage at the nt 101 site. Lowering the cellular level of RNase J1 results in less cleavage at this site and a 2-fold increase in half-life. Altering the sequence around nt 101 to contain only G and C residues (NotI RNA) completely inactivates this site as an RNase J1 target, and turnover of NotI RNA must then rely on one or more alternative pathways, most likely including one that involves RNase J1 5′-exonuclease activity. The 5′-SL structure presents a partial barrier to RNase J1 exonuclease activity such that turnover is slowed. However, this secondary exonucleolytic pathway is unaffected by lowering RNase J1 levels (cf. half-life of NotI RNA in wild-type and RNase J1 mutant strains) because the K_m for the RNase J1 exonucleolytic reaction is much lower than for the endonucleolytic reaction (11) and is therefore insensitive to the 5-fold decrease in cellular RNase J1 concentration. Addition of the 5′-SSS to trp leader RNA does not affect the dominant pathway for decay initiation that involves RNase J1 cleavage at nt 101 because the target site can be accessed independently of the 5′ end (see below). However, in 5′-SSS + NotI RNA, the combination of the 5′-SSS, which inhibits the exonucleolytic decay pathway, and the NotI site at nt 101, which eliminates the primary endonucleolytic decay pathway, results in a slower turnover rate in the wild-type strain that is exacerbated further in the RNase J1-deficient strain. The fact that lowering RNase J1 concentration has a stabilizing effect even in the case of 5′-SSS + NotI RNA (Fig. 2A, second row) suggests that turnover of this RNA may be via a secondary RNase J1 endonucleolytic target, e.g. at nt 40 (see below).

Cleavage at Nt 101 of 5′-SSS RNA—Biochemical studies of an RNase J enzyme from Thermus thermophilus indicated that there is a single catalytic site for both exonucleolytic and endonucleolytic cleavage (10). This site is immediately adjacent to a 5′-end-binding site, which accommodates a 5′-monophosphate end but apparently not a 5′-triphosphate end. This is the expected arrangement for the 5′-exonucleolytic activity of RNase J1 and explains why RNase J1 can degrade 5′-monophosphorylated RNA exonucleolytically but not 5′-triphosphorylated RNA (7, 10). However, it has not been clear how this relates to the endonucleolytic activity of RNase J1, which is apparently insensitive to the phosphorylation state of the 5′ end (7).

The in vivo results with 5′-SSS RNA, showing that half-life was unaffected by the presence of a strong structure at the 5′ terminus, suggested that RNase J1 endonucleolytic cleavage at nt 101 could occur independently of the 5′ end. To examine this, we first determined the in vivo RNase J1 cleavage site in 5′-SSS RNA, using a 3′-proximal probe in a Northern blot analysis to detect the 3′ fragment that results from cleavage. As can be seen in Fig. 3A, the identical 3′ fragment was detected for wild-type trp leader RNA and 5′-SSS RNA, whereas this fragment was not detected for NotI RNA and 5′-SSS + NotI RNA. These results indicated that RNase J1 was capable of trp leader RNA cleavage at its native internal target site, even when the RNA 5′ end was protected by a stem-loop structure.

We next examined the rate of RNase J1 cleavage of trp leader RNA in vitro. trp leader RNA and 5′-SSS RNA were synthesized to contain a labeled triphosphate 5′ end, which would leave the RNA sensitive to RNase J1 endonucleolytic activity but resistant to 5′-exonucleolytic activity. To increase the yield of in vitro transcription, which is limited because of the low concentration of
5′ End-independent RNase J1 Cleavage

The presence of a strongly structured moiety at the 5′ end of 5′-SSS RNA interfered with the RNase J1 5′-exonuclease activity on 5′-monophosphorylated RNA substrates. Release of 32P-labelled GMP was assayed by thin-layer chromatography. Solid squares, trp leader RNA; open circles, trp leader RNA with unstructured 5′-terminal region; open triangles, 5′-SSS RNA.

Initiating nucleotide needed to get significant incorporation of [γ-32P]NTP at the 5′ end, two (for 5′-SSS RNA) or three (for trp leader RNA) guanosine residues were added to the 5′ end. Incubation of trp leader RNA with RNase J1 resulted in a gradual disappearance of full-length substrate (140 nt) and appearance of a ~100-nt band that was the 5′ product of cleavage at nt 101 (Fig. 3B). A smaller band of ~40 nt was also detected, as we have observed previously (7), which represents weak RNase J1 cleavage at a site located in the upstream edge of the TBS. Incubation of 5′-SSS RNA with RNase J1 gave the expected 5′ cleavage product of ~130 nt (Fig. 3B). Smaller bands of ~70 and ~30 nt were also present. Importantly, the rate of cleavage for both of these substrates was nearly identical (Fig. 3C), demonstrating that RNase J1 endonuclease activity is insensitive to the presence of a strongly structured moiety at the 5′ end.

Exonucleolytic Attack from 5′ End—To demonstrate directly that the 5′-terminal structure in 5′-SSS RNA was affecting interaction of RNase J1 with the 5′ end, we examined the initiation of 5′-exonuclease activity on three versions of trp leader RNA that had a labeled 5′-monophosphate end. The three RNAs contained at their 5′ ends: 1) the wild-type 5′-SL sequence, with a predicted ΔG0 of ~9.3 kcal mol⁻¹; 2) the stem-loop sequence as in 5′-SSS RNA, with a predicted ΔG0 of ~11.1 kcal mol⁻¹; and 3) a mutated 5′-SL sequence such that no structure could form, with a predicted ΔG0 of ~0.8 kcal mol⁻¹. The latter substrate was made on a template that had six residues from the upstream side of the 5′-SL sequence mutated to their complement (see boxed nucleotides in Fig. 1A). In each case, the transcript began with three G residues to enhance transcript initiation at a site located in the upstream edge of the TBS. Incubation of 5′-SSS RNA with RNase J1 gave the expected 5′ cleavage product of ~130 nt (Fig. 3B). Smaller bands of ~70 and ~30 nt were also present. Importantly, the rate of cleavage for both of these substrates was nearly identical (Fig. 3C), demonstrating that RNase J1 endonuclease activity is insensitive to the presence of a strongly structured moiety at the 5′ end.

Involvement of RNase Y in trp Leader RNA Decay—When our model for RNase J1-mediated decay of trp leader RNA was first formulated, we were not aware of the existence of another essential B. subtilis endoribonuclease, RNase Y (18, 19). There are indications that RNase Y, like RNase E in E. coli, is a central element of an RNA degradosome complex that includes RNase J1 and PNPase (18, 32). We investigated the effect of reducing RNase Y expression on trp leader RNA decay, using a strain in which RNase Y expression is under IPTG-inducible control and is grown in 1 mM IPTG, as above for the RNase J1 mutant strain.

Half-lives of the four trp leader RNA constructs shown in Fig. 2A were determined in the RNase Y-mutant strain (Fig. 2A, third row). The remarkable result was that, for all four constructs, there was no significant difference between the half-lives in the RNase Y mutant strain compared with the RNase J1 mutant strain (cf. p values in parentheses). We reason that it is unlikely that the half-life of trp leader RNA depends on the endonuclease activities of both RNase Y and RNase J1 acting at the nt 101 site. If either enzyme could cleave at this site, we would expect that the enzyme activities were present at a high enough level that lowering the concentration of one of them would not affect trp leader RNA half-life. It is more likely that RNase Y does not act directly on trp leader RNA. Furthermore, 5′-SSS+NotI RNA was extremely stable in the RNase Y mutant strain (Fig. 2A), with a half-life of 15.7 min. Our hypothesis to explain the extreme stability of 5′-SSS+NotI RNA in the RNase...
J1 mutant strain, which involves resistance to RNase J1 5′-exonuclease activity, cannot apply to RNase Y, as the latter enzyme has only endonuclease activity. Rather, the recent evidence that RNase Y exists in a degradosome complex that includes RNase J1 (18, 32) may suggest that the effect of depleting RNase Y on trp leader RNA decay is an indirect one, resulting from a negative effect on RNase J1 activity. We believe that the close correlation of half-lives for all four constructs in the RNase J1 and RNase Y mutant strains lends credence to this hypothesis. In addition, if our findings with trp leader RNA are at all representative of decay of other RNAs (see below), the possibility that lowering RNase Y concentration affects RNase J1 activity suggests caution in interpreting results of experiments performed with RNase Y-mutant strains, as these results may reflect an indirect effect on RNase J1, rather than a direct effect of RNase Y itself.

trp Leader RNA Half-life in RNase J2 Deletion Strain—If our hypothesis for the indirect effect of lowering RNase Y concentration on RNase J1 activity was correct, we predicted that a similar effect might be observed in a strain deleted for RNase J2, which exists primarily in a heterotetrameric complex with RNase J1 (13). Indeed, Condon and colleagues (13) found in vitro that the RNase J1-J2 complex functions more efficiently as an endonuclease than either enzyme alone. trp leader RNA half-life was measured in a strain that contained no RNase J2. The results in Fig. 2A showed a significant increase in half-life from 2.5 min in the wild-type strain to 4.3 min in the RNase J2 deletion strain (p value = 0.003). Although the half-life in the RNase J2 deletion strain was still significantly shorter than in the RNase J1 mutant strain (5.4 min), it was not significantly different from the half-life in the RNase Y mutant strain (Fig. 2A, bottom row; p value in parentheses). Thus, we measured a similar effect on trp leader RNA stability for strains defective for RNase Y or deleted for RNase J2 and a possible explanation for both cases is an indirect effect on RNase J1 activity.

Decay of trp Leader RNA in Absence of TRAP—Two potential problems with using trp leader RNA as a model for RNase J1-initiated RNA decay are as follows: 1) the presence of the TRAP complex bound just upstream of the RNase J1 cleavage site and 2) the absence of translation. To address the first issue, it was important to determine whether cleavage at nt 101 and the effect of lowering RNase J1 levels on trp leader RNA decay could be observed even in the absence of TRAP binding, thus allowing us to generalize what we find with trp leader RNA to other RNAs that are not bound by TRAP. However, we could not simply delete or alter the TBS sequence, as formation of the trp leader 3′-TT structure depends on TRAP binding. That is, in the absence of TRAP binding (when tryptophan is scarce), the alternative antiterminator structure forms (see highlighted nucleotides in Fig. 1A), resulting in transcriptional read-through into the trp operon genes. Only when tryptophan-activated TRAP binds to the TBS are the antiterminator nucleotides in the TBS obscured, allowing the downstream nucleotides to engage in an alternative base-pairing to form the 3′-TT structure. To study the characteristics of trp leader RNA decay in the absence of TRAP binding, it was necessary to replace the trp leader 3′-TT with a terminator that forms constitutively.

The trp leader RNA 3′-TT stem-loop sequence was replaced with a 3′-TT stem-loop sequence from a different gene, rpsO, while preserving the nucleotide sequences upstream and downstream of the stem-loop structure (Fig. 5A). The rpsO 3′-TT was chosen because its size and strongly structured nature are similar to that of trp leader 3′-TT (although the predicted stability for the rpsO terminator is significantly higher; Fig. 5A), and because we observed previously that turnover of the rpsO 3′-TT fragment occurs by RNase J1 5′-exonuclease activity, as is the case with the trp leader 3′-TT fragment (7). The transcript from the trp leader RNA construct containing the rpsO 3′-TT is called trps:rpsO-TT RNA. Because nucleotides in the rpsO sequence were not complementary to sequences in the TBS, we assumed that the transcription terminator structure of trps:rpsO-TT RNA forms constitutively. Indeed, preliminary Northern blot analysis revealed that trps:rpsO-TT gave a strong band of the expected size in the presence or absence of TRAP (data not shown). In addition, virtually no transcriptional read-through products were observed for trps:rpsO-TT RNA, unlike the case of trp leader RNA, for which read-through products that likely arise from transcription that escapes TRAP binding can be detected (cf. Fig. 5B). The size of trps:rpsO-TT RNA was slightly smaller than trp leader RNA (this can be seen even on the overexposure in Fig. 5B), which we believe was due to the multiple, consecutive U residues at the downstream side of the rpsO 3′-TT stem sequence (Fig. 5A), resulting in earlier transcription termination than occurs with the trp leader RNA 3′-TT sequence.

We determined first whether RNase J1 cleavage was occurring on trps:rpsO-TT RNA at the same position as on trp leader RNA. A 5′ probe, which would detect both RNAs, and trp- or rpsO-specific 3′ probes were used in Northern blot analyses to detect upstream and downstream products of RNase J1 cleavage. Note that long exposure times are required to detect the upstream 101-nt fragment because its 3′ end is unstructured and is subject to rapid 3′→5′ exonucleolytic decay by PNPase. The results with the 5′ probe in Fig. 5B show that a band of identical size, ~100 nucleotides, is present for both trp leader RNA and trps:rpsO-TT RNA. The results with the 3′ probes in Fig. 5C show that the major 3′ fragment detected for trp leader RNA is around 40 nucleotides, whereas the major 3′ fragment detected for trps:rpsO-TT RNA is several nucleotides shorter, running below the 39-nt marker fragment. As stated above, we believe this 3′-terminal fragment is shorter because transcription termination occurs earlier with trps:rpsO-TT RNA. Thus, RNase J1 cleaves trps:rpsO-TT RNA at the same site as trp leader RNA.

Next, we measured the half-life of trps:rpsO-TT RNA in wild-type and RNase J1 conditional mutant strains (Table 1). The trps:rpsO-TT RNA half-life in the wild-type strain (3.6 min) was somewhat higher than what we found for trp leader RNA (2.5 min). Although the RNase J1 cleavage that we hypothesize is dominant in determining trp leader RNA half-life occurs at the same position in trps:rpsO-TT RNA as in trp leader RNA (Fig. 5, B and C), it is possible that the rate of such cleavage may be lower for trps:rpsO-TT. It is also possible that there is a stabilizing effect of the stronger 3′-TT structure of rpsO, which could provide better protection against a minor 3′ exonuclease
decay pathway from the 3' end (8). In the RNase J1 mutant strain, \textit{trp}:\textit{rpsO}-TT RNA half-life increased 2.3-fold to 8.2 min (Table 1), matching almost exactly the 2.2-fold increase in half-life observed for \textit{trp} leader RNA in the RNase J1 mutant strain (Fig. 2A). Similarly, a \textit{trp}:\textit{rpsO}-TT RNA derivative that had the NotI sequence at nt 101 gave a 3.1-fold increase in half-life to 11.0 min (Table 1, NotI: \textit{rpsO}-3'-TT RNA), not as large as the 4.1-fold increase we observed for \textit{trp} leader RNA (Fig. 2A) but substantial enough to conclude that RNase J1 cleavage at the nt 101 site is the major decay determinant also for \textit{trp}:\textit{rpsO}-TT RNA.

To measure \textit{trp}:\textit{rpsO}-TT RNA half-life in the absence of TRAP binding, strains were constructed that had the chromosomal \textit{mtrB} locus replaced with a plasmid-borne \textit{mtrB} gene deleted. It was gratifying to find that the half-lives in the wild-type and RNase J1 conditional mutant strains were unaffected by the absence of TRAP (Table 1). We also probed for the turnover of \textit{trp}:\textit{rpsO}-TT RNA 3' end-containing decay intermediates in the wild-type and RNase J1-depleted strains, with or without TRAP present. Below the blot are the amount of 3' terminal fragments, relative to the amount of full-length RNA in each lane. The marker lane is as described in the legend to Fig. 2.

### TABLE 1

\textit{trp}:\textit{rpsO}-TT RNA half-lives in various strains

| \textit{trp}:\textit{rpsO}-TT RNA | RNase J1 wild-type | RNase J1 mutant |
|-------------------------------|--------------------|-----------------|
| \textit{mtrB}^+               | 3.6 ± 0.4          | 8.2 ± 0.3       |
| \textit{mtrB}^-               | 11.0               | 7.7 ± 0.4       |

Below the blot are the amount of 3' terminal fragments, relative to the amount of full-length RNA in each lane. The marker lane is as described in the legend to Fig. 2.

FIGURE 5. Characteristics of \textit{trp}:\textit{rpsO}-TT RNA decay. A, nucleotide sequence of 3' -TT for \textit{trp} leader RNA and \textit{trp}:\textit{rpsO}-TT RNA. Predicted structures are shown, with the calculated ΔG° indicated below. Uncertainty about the actual 3' end of \textit{trp}:\textit{rpsO}-TT RNA is indicated by the parentheses. B, Northern blot analysis (3' -proximal probe) of RNase J1 5' cleavage product, indicated by asterisk at right. Migration of full-length (FL) and read-through (RT) RNA is indicated. The blot is overexposed to show the rapidly degraded 5' cleavage product. Marker lane (M) is as described in the legend to Fig. 2. C, Northern blot analysis of RNase J1 3' cleavage product, using \textit{trp}- and \textit{rpsO}-specific 3' probes. Major 3' terminal fragments are marked with an asterisk. Marker lanes (M) are as described in the legend to Fig. 2. D, accumulation of \textit{trp}:\textit{rpsO}-TT RNA 3' end-containing decay intermediates in wild-type and RNase J1-depleted strains, with or without TRAP present. Below the blot are the amount of 3' terminal fragments, relative to the amount of full-length RNA in each lane. The marker lane is as described in the legend to Fig. 2.

To measure \textit{trp}:\textit{rpsO}-TT RNA half-life in the absence of TRAP binding, strains were constructed that had the chromosomal \textit{mtrB} locus replaced with a plasmid-borne \textit{mtrB} gene deleted. It was gratifying to find that the half-lives in the wild-type and RNase J1 conditional mutant strains were unaffected by the absence of TRAP (Table 1). We also probed for the turnover of \textit{trp}:\textit{rpsO}-TT RNA 3' end-containing decay intermediates in the wild-type and RNase J1 mutant strains, in the presence or absence of TRAP. In the case of \textit{trp} leader RNA, such decay intermediates accumulate in the RNase J1 mutant strain (7). The results (Fig. 5D) showed about a 2-fold greater abundance of 3' end-containing fragments in the RNase J1 mutant strain compared with the wild-type strain, with no significant difference between the presence or absence of TRAP. Thus, all measures indicate that TRAP binding is irrelevant to the turnover of \textit{trp} leader RNA, and conclusions about the RNase J1 specificity in the decay pathway for \textit{trp} leader RNA may be valuable for understanding decay of other RNAs. The second potential problem of using \textit{trp} leader RNA as a model, i.e. the absence of translation, will be addressed in future experiments.

Block to PNPase Activity at GC-rich Sequence — The availability of \textit{trp}:\textit{rpsO}-TT RNA, which is produced in the absence of
TRAP binding, allowed us to reexamine a striking observation we had made with a trp leader RNA derivative called "NotI_34 RNA," in which nt 34 of trp leader RNA is replaced with the NotI GC sequence (Fig. 6A). Insertion of the NotI sequence upstream of the TBS had no effect on half-life but led to the dramatic accumulation of a 5' proximal fragment of ~50 nt (8) (cf. Fig. 6B, lane 1). We hypothesized that accumulation of this RNA fragment was because the inserted NotI site caused stalling of PNPase, which initiates 3' exonucleolytic degradation from the RNase J1 cleavage site at nt 101 (5). This is noteworthy, as PNPase appears to be otherwise insensitive to structures or sequences in mRNA decay intermediates (15). Using trp leader RNA, we could not compare accumulation of the 50-nt fragment in strains with and without PNPase because, in the absence of PNPase, TRAP is trapped on the TBS and a fragment accumulates whose 3' end is downstream of the TBS (5; see Fig. 6B, lane 2, caret). This fragment likely arises from the activity of 3' exonucleases other than PNPase that initiate at the RNase J1 cleavage site and at the 3' end of multiple readthrough products (see upper portion of Fig. 6B, lane 2). The validation of trprpsO-TT RNA as a surrogate for trp leader RNA allowed us to test the role of PNPase in accumulation of the 50-nt fragment.

A derivative of trprpsO-TT RNA was made that had the NotI site inserted at nt 34, which is called "NotI_34:trpsO-TT RNA." As a preliminary control, we determined whether the presence of the rpsO 3'-TT affected binding of TRAP to the TBS. A TRAP binding assay was performed as described under "Experimental Procedures." The data in Table 2 show that the apparent dissociation constants, K_{app}, for TRAP binding to trp leader RNA and trprpsO-TT RNA were very similar. This was also the case for NotI_34 RNA and NotI_34:trpsO-TT RNA, which both had a higher K_{app}, presumably due to the inserted GC sequence.

In a wild-type strain, full-length NotI_34:trpsO-TT RNA was observed, as well as a strong accumulation of a 50-nt fragment (Fig. 6B, lane 3). The involvement of PNPase in accumulation of the 50-nt fragment was demonstrated in a PNPase deletion strain (33) carrying NotI_34:trpsO-TT RNA (Fig. 6B, lane 4). 10-fold less of the 50-nt fragment was observed in this strain, demonstrating that the 50-nt fragment in the wild-type strain was indeed due to a block to PNPase processivity. The small amount of the 50-nt fragment that is observed presumably comes from a partial block to 3' degradation by other 3'-exonucleases (15). A weak ~110-nt band is also observed, which likely represents a block at the downstream edge of the TBS to other 3'-exonucleases that initiate decay at the RNase J1 cleavage site. As there is little read-through product for NotI_34:trprpsO-TT RNA, less of this fragment accumulates than in lane 2.

Finally, because transcription termination of NotI_34: rpsO-TT RNA was not dependent on TRAP, we could examine the possible requirement for TRAP binding on accumulation of the 50-nt fragment. In a strain that contained PNPase but no TRAP, accumulation of the 50-nt fragment was decreased 3-fold (Fig. 6B, compare lanes 3 and 5). We hypothesize that the block to PNPase activity caused by the NotI sequence at nt 34 is enhanced by the slowing of PNPase as it degrades through the TRAP-bound portion of the RNA. The amount of 50-nt fragment in the strain containing neither PNPase nor TRAP (Fig. 6B, lane 6) was similar to that in the strain containing TRAP but no PNPase (Fig. 6B, lane 4).

We have shown that the mechanism of trp leader RNA decay does not depend on TRAP binding, suggesting that our finding that RNase J1 endonuclease cleavage of trp leader RNA can occur by direct access may be generally true for other RNA targets of RNase J1. It is yet to be determined whether the endonuclease or exonuclease activity of RNase J1 is more important for RNA turnover in B. subtilis (11).
although the exonucleolytic decay pathway is obviously 5’ end-dependent, a decay pathway that begins with an endonucleolytic cleavage may be 5’ end-independent. Thus, a wide range of RNAs could be targeted for initiation of decay by RNase J1 whether or not the 5’ end was accessible.

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