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Decay of a Model mRNA in *Bacillus subtilis* by a Combination of RNase J1 5′ Exonuclease and RNase Y Endonuclease Activities▽†

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The involvement of the recently characterized 5′ exonuclease activity of RNase J1 and endonuclease activity of RNase Y in the turnover of Δ*ermC* mRNA in *Bacillus subtilis* was investigated. Evidence is presented that both of these activities determine the half-life of Δ*ermC* mRNA.

The 260-nucleotide (nt) Δ*ermC* mRNA (Fig. 1A) has served as a useful model for determining the effects of structural and translational elements on mRNA stability in *Bacillus subtilis* (1, 16, 17, 20). The involvement of the essential RNase J1, a bifunctional enzyme that has both endonuclease and 5′ exonuclease activities (10, 12), in Δ*ermC* mRNA decay was reported previously (20): the half-life of Δ*ermC* mRNA increased 2.5-fold, from 7.5 min to 18.5 min, in an RNase J1-limited strain. The terms "RNase J1-" and "RNase Y-limited strain" refer to strains in which expression of the particular RNase is under p_{spac} promoter control, which results in an RNase concentration severalfold lower than that in the wild-type strain (7). A 130-nt derivative of Δ*ermC* mRNA, which had an in-frame deletion of nt 87 to 215 and which we call here Δ*ermC*-del (Fig. 1A), showed a 2-fold increase in half-life in the wild-type strain (14.6 min). This result indicated strongly that exonucleolytic degradation by RNase J1 from the 5′ end could not account completely for Δ*ermC* mRNA turnover, as this mode of decay was unlikely to be affected by an internal deletion. We hypothesized that one or more RNase J1 endonuclease cleavage sites were located between nt 87 and 215 and that deletion of this sequence increased half-life by eliminating these decay initiation sites. Whether exonucleolytic degradation by RNase J1 from the 5′ end also contributed to determining the half-life of the native transcript was not investigated. At the time of this study, we were unaware of the existence of RNase Y, a recently discovered essential endoribonuclease (5, 15) that may be even more important than RNase J1 for turnover of bulk mRNA (15). Here, we address the roles of RNase J1 5′ exonuclease activity and RNase Y endonuclease activity in Δ*ermC* mRNA decay.

The effect of RNase Y on Δ*ermC* mRNA half-life was determined in an RNase Y-limited strain (15). Northern blot analysis, performed as described previously (19), showed that reduction of RNase Y levels resulted in an almost 2-fold increase in Δ*ermC* mRNA half-life (7.5 min to 13.8 min; Table 1). Thus, in addition to RNase J1, RNase Y is also involved in determining Δ*ermC* mRNA stability.

We next measured the effect of RNase Y on the half-life of Δ*ermC*-del mRNA and found that it was unchanged from that of the wild-type strain (14.6 min and 15.0 min; Table 1). This suggested that the deleted sequence (nt 87 to 215) in Δ*ermC*-del mRNA contained one or more RNase Y cleavage sites; deletion of this sequence gave an mRNA that was no longer targeted by RNase Y.

We showed previously that prominent Δ*ermC* mRNA decay intermediates of <140 nt could be detected with a 3′-proximal probe in the RNase J1-limited strain, although not in the wild-type strain (20). We hypothesized that these fragments were generated by endonuclease cleavages at around nt 120 and 175 and were degraded rapidly in the wild-type strain by RNase J1 in the 5′-to-3′ direction. The approximate locations for two cleavage sites are indicated in Fig. 1A, and the resultant decay intermediates are indicated by carets in Fig. 2. As the current results indicated that RNase Y was initiating decay by endonuclease cleavage between nt 87 and 215, we tested whether RNase Y was involved in generating these 3′ fragments. The levels of 3′-terminal decay intermediates were compared in strains that were RNase J1 depleted and expressed either wild-type or decreased levels of RNase Y (Fig. 2). The results showed that decreased RNase Y levels resulted in a decreased level of 3′-terminal decay intermediates. Thus, we concluded that RNase Y makes endonucleolytic, decay-initiating cleavages in the region between nt 87 and 215.

The half-life of Δ*ermC*-del mRNA, which was 2-fold greater than that of Δ*ermC* in the wild-type strain, was increased at least another 2-fold in the RNase J1-limited strain (to >30 min) (Table 1), suggesting that additional RNase J1 target sites outside the 87-to-215-nt region were present on Δ*ermC*-del mRNA (20). The endpoint of the 87-to-215-nt deletion is just three nucleotides upstream of the Δ*ermC* stop codon (Fig. 1A), which is followed immediately by an extremely strong transcriptional terminator stem-loop structure ($\Delta G_0 = -24.1$ kcal/mol). For this reason, it was considered unlikely that the se-

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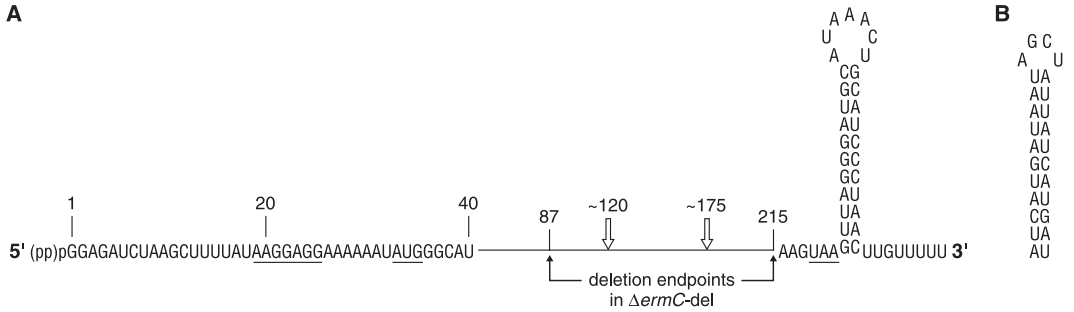

FIG. 1. (A) Schematic diagram of Δ*ermC* mRNA. Translational signals are underlined. Open arrows above the schematic show approximate positions of putative RNase Y cleavage sites that would generate the two major 3′-terminal decay intermediates indicated by carets in Fig. 2. The mRNA is shown with either a monophosphate or a triphosphate at the 5′ end. Endpoints of the deletion within Δ*ermC* mRNA that gave Δ*ermC*-del mRNA are shown. (B) Sequence and predicted structure of the 5′SS mRNA stabilizer.

quence downstream of the Δ*ermC*-del open reading frame (ORF) was a target site for RNase J1 cleavage. Rather, RNase J1 could cleave endonucleolytically at one or more sites between positions +1 and +87 (see below) or could degrade exonucleolytically from the 5′ end. To examine the latter possibility, a splinted ligation assay (PABLO) specific for 5′ ends bearing a single phosphate (3) was used to determine the level of monophosphorylation at the 5′ end of Δ*ermC* mRNA. We reasoned that a decrease in the level of 5′ exonucleolytic activity in the RNase J1-limited strain would result in an increase in the relative abundance of monophosphorylated 5′ ends, which are uniquely capable of undergoing ligation to an oligonucleotide in the PABLO assay. The results shown in Fig. 3 reveal that the ligation yield for Δ*ermC* mRNA, which was 27.4% in the wild-type strain, increased ~1.5-fold to 42.5% in the RNase J1-limited strain. (Note that the ligation yield depends not only on the percentage of 5′ ends that are monophosphorylated but also on the ligation efficiency. Ligation efficiencies are typically only 50% to 80% [4]. Thus, the ligation yields for Δ*ermC* mRNA *in vivo* likely reflect an even higher percentage of 5′-monophosphorylated mRNA.) The increase in ligation yield for Δ*ermC* mRNA in the RNase J1-limited strain is best explained by a reduction in 5′ exonuclease activity that removes the 5′-terminal, ligatable nucleotide. In addition, the reduction in RNase J1 activity should result in a larger amount of Δ*ermC* mRNA, and this is observed (Fig. 3, lane 3 versus lane 1).

A similar result was observed for Δ*ermC*-del mRNA, with a ligation yield of 42.7% in the wild-type strain that increased ~1.5-fold to 62.1% in the RNase J1-limited strain (Fig. 3), as well as an increased level of the transcript (Fig. 3, lane 7 versus lane 5). The high ligation yields for Δ*ermC*-del mRNA, relative to those of Δ*ermC* mRNA, in wild-type cells were expected since monophosphorylated Δ*ermC*-del mRNA is degraded more slowly and this should result in a higher ligation yield (see Fig. S1 in the supplemental material). We conclude that a substantial fraction of Δ*ermC* mRNA is 5′-monophosphorylated and that this intermediate is degraded by RNase J1, whose 5′ exonuclease activity is known to be blocked by a triphosphorylated 5′ end (9, 11, 14). Thus, the increased stability of Δ*ermC* and Δ*ermC*-del mRNAs in the RNase J1-limited strain is likely due to diminished exonucleolytic decay from the 5′ end. This conclusion is consistent with the prolonged lifetime of Δ*ermC* mRNA in cells lacking RppH, the enzyme that converts triphosphorylated 5′ ends to monophosphorylated 5′ ends (14).

To test whether access to the 5′ end was required for determining Δ*ermC* mRNA half-life, we constructed a derivative, called 5′SS-Δ*ermC* mRNA, which contained a 26-nt stabilizer sequence at the 5′ terminus (Fig. 1B). This sequence is capable of forming a strong secondary structure ($\Delta G_0 = -10.0$ kcal/

TABLE 1. Δ*ermC* mRNA half-lives in wild-type and RNase mutant strains

mRNA	Half-life (min)[a]		
	Wild type	J1 mutant	Y mutant
Δ*ermC*	7.5 ± 0.1	18.5 ± 2.8	13.8 ± 1.7
Δ*ermC*-del	14.6 ± 1.6	>30	15.0 ± 0.7
5′SS-Δ*ermC*	18.3 ± 1.3	14.9 ± 1.7	31.9 ± 2.8
5′SS-Δ*ermC*-del	>45		

[a] Values are the averages ± SD of results from three determinations.

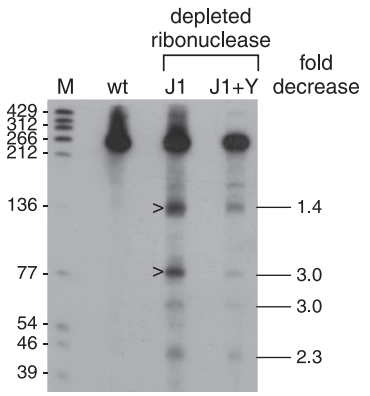

FIG. 2. Analysis of Δ*ermC* mRNA decay fragments probed with a 36-nt 3′-terminal probe that was complementary to Δ*ermC* mRNA nt 205 to 240. The strain types are indicated above each lane: wt, wild type; J1, RNase J1 limited; J1+Y, RNase J1 and RNase Y limited. Marker lane M contained 5′-end-labeled TaqI fragments of plasmid pSE420 DNA (2), with the sizes of these fragments indicated on the left. On the right is the fold decrease of each of four prominent decay intermediates in the J1+Y lane relative to the J1 lane and normalized to the amount of full-length RNA (average of results from four experiments).

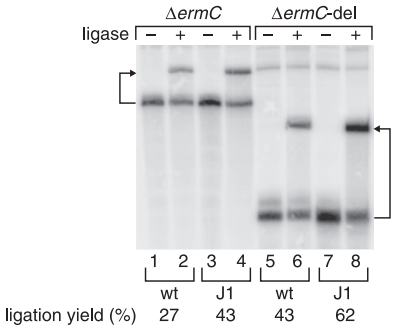

FIG. 3. PABLO analysis of the extent of 5′-monophosphorylation of Δ*ermC* and Δ*ermC*-del mRNAs in wild-type and RNase J1-limited cells. Details of the oligonucleotides used in the assay are as described previously (14). Arrows point from each transcript to its ligation product.

mol) and has been shown in other contexts to function as a 5′ stabilizer (13, 18). The addition of the 5′SS to Δ*ermC* mRNA substantially increased the half-life in the wild-type strain to 18.3 min (Table 1). The presence of a 5′-terminal structure is known to inhibit the pyrophosphohydrolase activity needed to convert the 5′-triphosphate end of a native transcript to a 5′-monophosphate end (8, 14); thus, the 5′ end of 5′SS-Δ*ermC* mRNA should be primarily triphosphorylated. Since RNase J1 5′ exonuclease activity is blocked by the presence of a 5′-triphosphate (9, 11, 14), we predicted that the half-life of 5′SS-Δ*ermC* mRNA would not be affected by lowering RNase J1 levels. This was indeed the case: the half-life of 5′SS-Δ*ermC* mRNA in the RNase J1-limited strain was 14.9 min (Table 1), not significantly different from the half-life in the wild-type strain (*P* value of 0.02). Thus, RNase J1 was not involved in determining the half-life of 5′SS-Δ*ermC* mRNA, suggesting that the 5′ end of Δ*ermC* is the major, if not only, decay-initiating target for RNase J1. By primer extension analysis of Δ*ermC* mRNA, we found previously in the RNase J1-limited strain a minor 5′ end at the +10 position (19), and it has been suggested that this may be due to endonucleolytic cleavage by a different RNase (6). We found that the amount of this "+10 band" decreased severalfold in the 5′SS-Δ*ermC* mRNA construct (data not shown), suggesting that it may represent a minor block to RNase J1 5′ exonuclease degradation.

On the other hand, we found that depletion of RNase Y significantly increased the half-life of 5′SS-Δ*ermC* mRNA. The half-life of 5′SS-Δ*ermC* mRNA increased from 18.3 min in the wild-type strain to 31.9 min in the RNase Y-limited strain (Table 1). This was likely due to decreased cleavage by RNase Y in the 87-to-215-nt region. A prediction of this hypothesis was that adding the same 5′ stem structure to Δ*ermC*-del mRNA, which would render it resistant to RNase J1 activity at the 5′ end, in addition to being devoid of internal RNase Y target sites, should result in an extremely stable mRNA, even in the wild-type strain. This prediction was verified, as shown in Table 1. There was virtually no decrease in the amount of 5′SS-Δ*ermC*-del mRNA, even at the latest time point (45 min).

Shahbabian and colleagues have reported that RNase Y activity is sensitive to the 5′ phosphorylation state, with 20-fold more activity *in vitro* on substrates containing a 5′-monophosphate end than a 5′-triphosphate end (15). Here, we find that the half-life of 5′SS-Δ*ermC* mRNA, whose 5′ end is likely to be substantially in the triphosphorylated form, was still dependent on RNase Y. This suggests that, *in vivo*, RNase Y does not absolutely require a 5′-monophosphate end for access to an internal cleavage site that is important for determining half-life.

In summary, the model Δ*ermC* mRNA has been used to obtain evidence for both RNase J1-mediated exonucleolytic and RNase Y-mediated endonucleolytic activities in the turnover of a single mRNA. We hypothesize that such cooperation is likely to occur in the decay of many *B. subtilis* mRNAs. Efforts to extend our findings from model RNAs to the *B. subtilis* transcriptome are under way.

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