Autoregulation of AbsB (RNase III) Expression in *Streptomyces coelicolor* by Endoribonucleolytic Cleavage of *absB* Operon Transcripts

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The *Streptomyces coelicolor* *absB* gene encodes an RNase III family endoribonuclease and is normally essential for antibiotic biosynthesis. Here we report that AbsB controls its own expression by sequentially and site specifically cleaving stem-loop segments of its polycistronic transcript. Our results demonstrate a ribonucleolytic regulatory role for AbsB in vivo.

Streptomycetes are a family of prokaryotic soil-dwelling microorganisms whose morphologically and developmentally complex life cycle involves mycelial growth and spore formation (8). These eubacteria also are notable for their production of pharmacologically useful compounds, including antitumor agents, immunosuppressants, and more than two-thirds of all natural antibiotics (6, 9). *Streptomyces coelicolor*, which has been the most widely studied streptomycete, synthesizes four identified antibiotics (9), whose production is controlled during the *S. coelicolor* life cycle by the combined actions of a series of regulatory and biosynthetic-pathway genes (4).

The *absB* gene was discovered in a screen for *S. coelicolor* mutants that show defective antibiotic synthesis but normal morphological development (1). Recently, however, AbsB has been found also to be required for the proper formation of sporulation septa (14). Subsequent sequence analysis revealed that the N-terminal domain of the AbsB protein contains a motif characteristic of RNase III family endoribonucleases (13). Members of this enzyme family, which has been implicated in the processing of pre-rRNA, rRNA, polycistronic mRNAs, and small regulatory RNAs (3, 10), normally cleave duplex segments of RNAs configured as stem-loop structures and are ubiquitous among prokaryotes, eukaryotes, and archaean (10). The originally isolated point mutation in AbsB substitutes a proline for a leucine in the C-terminal end of the protein (13). That AbsB may, in fact, have ribonucleolytic activity was first suggested by the accumulation of 30S rRNA precursors in *absB* mutant bacteria (13); later work has shown that purified AbsB protein can cleave transcripts encoded by the *S. coelicolor* *rpsO-pnp* operon in vitro (7). However, there has been no direct evidence in vivo of RNase function carried out by the AbsB protein.

The *S. coelicolor absB* gene is predicted to be the terminal open reading frame (ORF) of a three-gene operon that also encodes a putative RNase III family endoribonuclease (SC7A1.14, SCO5570) and RmpF, a homologue of ribosomal protein L32 (SCO5571) (13, 14). DNA microarray results showed increased *absB* transcript abundance in bacteria mutated in *absB* (11), suggesting that the AbsB protein may be targeting its own transcript ribonucleolytically. Consistent with this notion, Northern blot analysis shows the accumulation of an ~2,000-nucleotide (nt) RNA band corresponding in length to the full-length SC7A1.14-rmpF-*absB* transcript in *S. coelicolor* strain C120 (Fig. 1A), which contains a point mutation that substitutes a proline for the leucine at amino acid position 120 of the AbsB protein (13). Moreover, elevation of the steady-state level of 2,000-nt SC7A1.14-rmpF-*absB* transcripts was accompanied by a decreased rate of decay of these transcripts, as measured by the analysis of RNA samples taken at different times after rifampin addition, which prevents the initiation of new transcripts and consequently enables the determination of the rate of loss of previously synthesized mRNA. As shown in Fig. 1B, the half-life of SC7A1.14-rmpF-*absB*-encoded transcripts in the *absB* mutant C120 strain was approximately three times the half-life of such transcripts in the parental *absB*+ strain, J1501. Accumulation of SC7A1.14-rmpF-*absB* transcripts in *absB* mutant bacteria was reversed by adventitious expression of the cloned wild-type *absB* ORF under the control of the thiostrepton-inducible tipA promoter (Fig. 1C), as was the defect in antibiotic production. Such expression resulted in the production of an ~1,000-nt transcript corresponding in length to the *absB* ORF (Fig. 1C, lanes 6 and 8). Additionally, the appearance of two smaller RNA transcripts (750 and 200 nt) in cells overexpressing the *absB* ORF sequence suggested that the ORF transcript contains a site targeted by the AbsB protein.

To elucidate more precisely the sites of ribonucleolytic cleavage of SC7A1.14-rmpF-*absB* transcripts by AbsB, we produced hexahistidine-tagged wild-type and mutant AbsB proteins in protease-deficient *Escherichia coli* strain BL21 by cloning the *absB* ORF in expression plasmid pET28a and purified these proteins by Ni column chromatography (2). While both proteins were produced in and purified from *E. coli* under identical conditions, Western blot analysis, using anti-His antibody, of the Ni column eluates obtained resulted in a fivefold greater yield of the mutant protein (data not shown). Additionally, we observed that the 1,000-nt transcript encoding the wild-type AbsB protein was cleaved in *E. coli* (Fig. 2A) to yield an ~750-nt fragment whereas the transcript encoding the mutant AbsB protein remained intact, suggesting that the AbsB
protein acts ribonucleolytically in vivo on absB mRNA synthesized in that microorganism. To identify the sites targeted by the AbsB protein, we amplified two segments of S. coelicolor genomic DNA corresponding to the full-length absB operon by PCR with forward primers that included the E. coli bacteriophage T7 promoter sequence and used the resulting amplicons as templates for the in vitro production of RNAs (RNA A, 1,877 nt; RNA B, 1,842 nt; Fig. 2D) generated by T7 RNA polymerase; these RNAs have identical 3′ termini but differ at their 5′ ends. As shown in Fig. 2B, treatment of either RNA A or RNA B with purified wild-type His-tagged AbsB yielded two prominent bands (II-1 and II-2) corresponding, respectively, to RNA species approximately 1,200 and 500 nt in length and two less prominent bands (I-1 and I-2) corresponding to 1,700- and 200-nt species (Fig. 2B, lanes 2 and 5). In contrast, only faint bands of various lengths were detected following treatment of the RNA with the mutant AbsB protein (Fig. 2B, lanes 3 and 6), consistent with the defective ribonucleolytic activity observed in vivo in absB mutant bacteria. Additional experiments in which RNA A was incubated for various lengths of time with the wild-type enzyme (Fig. 2C) showed (i) that cleavage of RNA A was time dependent, (ii) that the abundance of band I-1 reached a maximum after 10 min of incubation and decreased subsequently, and (iii) that RNA bands II-1 and II-2 first appeared after 10 to 15 min of incubation and reached maximal abundance after 60 min of incubation. Taken together, these results argue strongly that SC7A1.14-rmpF-absB transcripts are cleaved sequentially at two separate sites and that RNA species I-1 is a decay intermediate that is subsequently cleaved to generate the shorter RNA species, which is not degraded further by AbsB.

The lengths of the cleavage products observed following the treatment of RNA A and RNA B with the AbsB protein suggest that the site of initial cleavage of the polycistronic SC7A1.14-rmpF-absB transcript is within the absB ORF, whereas the second cleavage event occurs within SC7A1.14 (Fig. 2D). As the 3′ termini of RNA A and RNA B are identical but RNA A is initiated 36 nt 5′ to RNA B (Fig. 2D) generated by T7 RNA polymerase; these RNAs in vitro production of RNAs (RNA A, 1,877 nt; RNA B, 1,842 nt; Fig. 2D) generated by T7 RNA polymerase; these RNAs have identical 3′ termini but differ at their 5′ ends. As shown in Fig. 2B, treatment of either RNA A or RNA B with purified wild-type His-tagged AbsB yielded two prominent bands (II-1 and II-2) corresponding, respectively, to RNA species approximately 1,200 and 500 nt in length and two less prominent bands (I-1 and I-2) corresponding to 1,700- and 200-nt species (Fig. 2B, lanes 2 and 5). In contrast, only faint bands of various lengths were detected following treatment of the RNA with the mutant AbsB protein (Fig. 2B, lanes 3 and 6), consistent with the defective ribonucleolytic activity observed in vivo in absB mutant bacteria. Additional experiments in which RNA A was incubated for various lengths of time with the wild-type enzyme (Fig. 2C) showed (i) that cleavage of RNA A was time dependent, (ii) that the abundance of band I-1 reached a maximum after 10 min of incubation and decreased subsequently, and (iii) that RNA bands II-1 and II-2 first appeared after 10 to 15 min of incubation and reached maximal abundance after 60 min of incubation. Taken together, these results argue strongly that SC7A1.14-rmpF-absB transcripts are cleaved sequentially at two separate sites and that RNA species I-1 is a decay intermediate that is subsequently cleaved to generate the shorter RNA species, which is not degraded further by AbsB.

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tional in vitro experiments, truncated RNAs that individually contain sequences of each proposed AbsB target site were cleaved at corresponding locations (data not shown), indicating that the site specificity of AbsB-mediated cleavages is determined by the RNA sequence in the region of cleavage rather than by interactions between distant regions of the full-length operon transcript.

Primer extension analyses of intact, AbsB-cleaved SC7A1.14-rmpF-absB transcripts precisely mapped the two cleavage sites identified by the above in vitro experiments (Fig. 3A for site I and B for site II). The detection of faint bands representing apparent cleavage products following treatment of the substrate with the mutant protein suggests that the leucine-to-proline substitution at amino acid position 120 does not totally abolish ribonucleolytic activity (Fig. 3A, lane 3). Specific primer extension products were observed only after the treatment of substrates with the wild-type AbsB protein (Fig. 3A and B, lanes 2). Additional primer extension analyses with total RNA isolated from the wild-type strain (J1501) as the template showed bands of the same lengths as those generated by AbsB cleavage of its transcript in vitro (Fig. 3C, lanes 1 and 3), whereas no such bands were detected in absB mutant strain C120 (Fig. 3C, lanes 2 and 4), confirming that AbsB cleaves at the same two sites in vivo and also confirming that the in vivo ribonucleolytic action of the mutant AbsB protein is dramatically impaired. Cleavages at both sites occurred in regions predicted to form stem-loop structures by Mfold analysis (Fig. 3D) (15), as is characteristic of cleavages by RNase III family enzymes (10).

There is no obvious sequence homology between these two AbsB cleavage sites in the SC7A1.14-rmpF-absB transcript and the site in the transcript of the intergenic region of the rpoS-pnp operon found previously to be cleaved by the AbsB protein in vitro (7). However, both AbsB cleavages in SCA7A1.14-rmpF-absB occurred within the internal single-stranded regions of stem-loop structures, as did the cleavage of the rpoS-
FIG. 3. Mapping of SC7A1.14-\textit{rmpF-absB} operon cleavage sites by primer extension. Substrates were either 20 ng RNA synthesized as described above and treated with AbsB protein in vitro or 100 μg total \textit{S. coelicolor} RNA. Primer I (5'-CGCCTGTCGGGGTCCGCGT-3'), a synthetic deoxyribonucleotide corresponding to the sequence -150 nt 3' to site I (as shown in Fig. 2D) and primer II (5'-GGTCGTCCGACAGGGCGCAC-3'), located -150 nt 3' to site II, were end labeled with [γ-32P]ATP by using T4 polynucleotide kinase. A Primer Extension System (Promega) and a 7-deaza-dGTP sequencing kit (USB) were used for the extension reactions (left) and the generation of sequencing ladders (right). (A) Uncleaved RNA A (lane 1), RNA A treated with AbsB (lane 2), and RNA A treated with mutant AbsB protein (lane 3) were reverse transcribed with primer I. The products were separated on 10% denatured polyacrylamide gel and exposed to a PhosphorImager. (B) Primer extension analysis of cleavage site II. RNA A treated as described for panel A was reverse transcribed with primer II. (C) A 100-μg sample of RNA from the wild-type (lanes 1 and 3) or \textit{absB} mutant (lanes 2 and 4) strain was reverse transcribed with the primers for sites I and II. No RNA was added to the gel lanes located between lanes 2 and 3. (D) Secondary structures of the RNA sequences of these two cleavage sites identified. The positions where AbsB cleaves are indicated. The molecular marker sizes beside the gels are in nucleotide bases.
pnp transcript by AbsB, which resembles cleavage by Bacillus subtilis RNase III rather than by E. coli RNase III, which normally cleaves within a base-paired hairpin segment (10, 12).

Our results show that AbsB autoregulates its expression by cleaving its own transcript and that an important consequence of the mutational ablation of absB activity, which results in the absence of antibiotic biosynthetic functions, is the loss of cleavage ability. The loss of cleavage ability in absB mutant bacteria implies that the previously observed decrease in the steady-state level of the multiple mRNAs in these bacteria (11) is a secondary rather than a primary effect of AbsB cleavage.

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REFERENCES