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Control of Expression of the RNases J1 and J2 in *Bacillus subtilis*

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In *Bacillus subtilis*, the dual activity 5′ exo- and endoribonucleases J1 and J2 are important players in mRNA and stable RNA maturation and degradation. Recent work has improved our understanding of their structure and mechanism of action and identified numerous RNA substrates. However, almost nothing is known about the expression of these enzymes. Here, we have identified the transcriptional and translational signals that control the expression of the *rnjA* (RNase J1) and *rnjB* (RNase J2) genes. While the *rnjB* gene is transcribed constitutively from a sigma A promoter, optimal expression of RNase J1 requires cotranscription and cotranslation with the upstream *ykzG* gene, encoding a protein of unknown function. In the absence of coupled translation, RNase J1 expression is decreased more than 5-fold. Transcription of the *ykzG* operon initiates at a sigma A promoter with a noncanonical −35 box that is required for optimal transcription. Biosynthesis of RNase J1 is autocontrolled within a small range (1.4-fold) and also slightly stimulated (1.4-fold) in the absence of RNase J2. These controls are weak but might be useful to maintain the overall RNase J level and possibly also equimolar amounts of the two nucleases in the cell that primarily act as a heterodimer *in vivo*.

RNases J1 (*rnjA*) and J2 (*rnjB*) play an important role in the maturation and degradation of RNA in *Bacillus subtilis* (1). These paralogous enzymes share 70% amino acid similarity and were initially characterized as endoribonucleases with *Escherichia coli* RNase E-like cleavage specificity (2). In addition, RNase J1 has a robust 5′-3′ exoribonuclease activity (3), but the equivalent activity of RNase J2 is almost 2 orders of magnitude weaker (4). RNases J1 and J2 were thus the first known enzymes with dual exo- and endoribonucleolytic activities, both of which are thought to be performed by a single catalytic site (5). The two enzymes copurified in stoichiometric quantities (2), estimated at about 2,500 copies/cell and forming a heterodimeric complex which can alter cleavage site preference compared to the individual enzymes (4). RNases J1 and J2 have been suggested to be part of a larger degradosome assembly centered around RNase Y (6, 7), but this association remains a subject of debate as it has not been reproduced in other studies (4, 8).

Even though global mRNA stability is only weakly affected by RNases J1 and J2 (2), these enzymes play an extensive role in the maturation and degradation of many mRNAs (9–13), as well as the maturation of rRNA (14) and small cytoplasmic RNA (15).

Despite their importance in RNA metabolism, there was no information on how the expression of these RNases is controlled. Here, we have identified major elements that direct and influence the expression of the *B. subtilis rnjA* and *rnjB* genes, i.e., transcription and translational signals and genetic context.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *B. subtilis* strains used in this work are derivatives of strain SSB1002, a wild-type laboratory stock strain derived from strain 168. *E. coli* strain JM109 was used for plasmid constructions, and *E. coli* XL1-Blue was used as a host for site-directed mutagenesis experiments. *B. subtilis* and *E. coli* strains were grown at 37°C in LB medium. When required, the following antibiotics were added to the medium: kanamycin (5 μg/ml), chloramphenicol (4 μg/ml), and spectinomycin (100 μg/ml) for *B. subtilis* and ampicillin (200 μg/ml) for *E. coli*. In *B. subtilis* strain SSB356, the *rnjA* gene is under the control of the xylose-inducible P_{xyl} promoter (9). Depletion of RNase J1 was achieved by growing the cells without inducer until a decrease in growth rate became perceptible.

Plasmid constructs. (i) pHM14. pHM14 is the vector for the construction of translational *lacZ* fusions to be integrated in single copy into the *B. subtilis amyE* locus. The transcriptional *lacZ* fusion vector pHM2 (16) was converted to a translational fusion vector by replacing an 0.9-kb EcoRI-ClaI fragment with an 0.8-kb EcoRI-ClaI fragment from plasmid pAC7 (17).

(ii) pHMJ8. Plasmid pHMJ8 contains a translational *rnjA-lacZ* fusion. A 380-bp PCR fragment (primers HP900 and HP901; see Fig. 2) comprising a region starting 118 nucleotides (nt) upstream of the *ykzG* open reading frame (ORF) and ending with a 5′-terminal *rnjA* sequence encoding the first 16 amino acids was inserted as an EcoRI-BamHI fragment into the respective sites of plasmid pHM14.

(iii) pHMJ32. pHMJ32 is a short transcriptional *ykqZ/rnjA-lacZ* fusion. A 105-nt PCR fragment (primers HP900 and HP1217; see Fig. 2) was cleaved with EcoRI-BamHI and inserted into the respective sites of the *lacZ* fusion vector pHM2 (16).

(iv) pHMJ33. pHMJ33 is a long transcriptional *ykqZ/rnjA-lacZ* fusion. A 459-nt PCR fragment (primers HP899 and HP1217; see Fig. 2) was cleaved with EcoRI-BamHI and inserted into the respective sites of the *lacZ* fusion vector pHM2 (16).

(v) pHMJ38. pHMJ38 is a transcriptional *ykqZ-lacZ* fusion. A 48-nt fragment was generated by hybridizing complementary oligonucleotides HP1251 (AATTCGAATATAATAGAATGAGCGAGTCTAACATACGG CATTTAAGGA) and HP1252 (GATCCTCCTTAAATGCCGTATGTTA GACTCGCTCATTCTATTATATTC). The 5′ protruding ends were used for direct ligation of the DNA into the *lacZ* fusion vector pHM2 restricted with EcoRI and BamHI, respectively. The cloned fragment contains sequences from 6 nucleotides upstream of the −10 box to position −10 with respect to the *ykqZ* start codon (see Fig. 2).

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(vi) pHMJ40. pHMJ40 is a translational *rnjB-lacZ* fusion. A 151-nt PCR fragment (primers HP1224 and HP1225; see Fig. 5) was cleaved with EcoRI-BamHI and inserted into the respective sites of the *lacZ* fusion vector pHM14.

(vii) pHMJ43. pHMJ43 is a transcriptional *ykqZ/rnjA-lacZ* fusion. The same 380-bp PCR fragment (primers HP900 and HP901; see Fig. 2) used for the construction of the translational *lacZ* fusion pHMJ8 was inserted as an EcoRI-BamHI fragment into the respective sites of the transcriptional *lacZ* fusion vector pHM2 (16).

RNA isolation. Total RNA was isolated by an adaptation of a previously published protocol (18). Briefly, a cell pellet from a 10- to 20-ml culture was washed in 1 ml TSE buffer (100 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM EDTA) and resuspended in 200 μl ice-cold STET buffer (50 mM Tris-HCl, pH 8, 8% sucrose, 0.5% Triton X-100, 10 mM EDTA) containing 3 mg/ml lysozyme and held on ice for 5 min. The samples were mixed with an equal volume of phenol-H_2O and heated for 1 min at 100°C. After phase separation, the aqueous phase was reextracted once with phenol (pH 8) and once with phenol-chloroform. The RNA was precipitated with ethanol-LiCl and resuspended in 50 μl water.

Primer extension assay. The primer was 5′ end labeled with [γ-^{32}P]ATP, hybridized to 5 μg of total RNA, and extended with the Superscript III reverse transcriptase (Invitrogen) as described by the manufacturer.

S1 nuclease mapping. Thirty micrograms of total RNA was mixed with 5′-end-labeled oligonucleotide HP859 (10^5 cpm, ~10 ng), precipitated, and resuspended in 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4), 1 mM EDTA (pH 8), 0.4 M NaCl, and 80% deionized formamide. Hybridization was carried out by incubating the mixture at 48°C overnight, and S1 nuclease (Fermentas) digestion (200 U) was carried out according to the manufacturer's instructions at 37°C for 30 min. The reaction products were resolved on a 5% polyacrylamide gel.

Site-directed mutagenesis. Promoter and initiation codon mutations were introduced on the pHMJ8 and pHMJ43 plasmids by the QuikChange strategy (Stratagene) using KOD DNA polymerase (Novagen).

β-Galactosidase measurements. For β-galactosidase measurements, cells were grown in the appropriate medium and 3 to 5 ml of sample of culture was analyzed as described elsewhere (19). All reported values are based on two to four independent measurements.

Western blot analysis. For Western blot analysis, 10 μg of protein extract was separated by one-dimensional SDS-PAGE (12.5%). After electrophoretic transfer of the proteins, the nitrocellulose membrane (GE Healthcare) was stained with amido black to check for equal transfer across all lanes. The membrane was blocked for 1 h with 5% skim milk powder in phosphate-buffered saline (PBS)–Tween buffer (100 mM NaH_2PO_4-Na_2HPO_4, pH 7.4, 100 mM NaCl, 0.1% Tween) and incubated with polyclonal RNase J1-J2 antibody dilutions in PBS-Tween for at least 4 h. After washing (4 times for 10 min each with PBS-Tween buffer), the membrane was incubated with a 1/10,000 dilution of protein A-^{125}I (Perkin-Elmer; 10 μCi/μg) in PBS-Tween buffer. After being washed with PBS-Tween, the blot was scanned using a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Quantification of the bands was carried out with the ImageQuant software (Molecular Dynamics).

RESULTS

***rnjA* is transcribed as a bicistronic transcript from a single promoter.** We determined the 5′ end of the *rnjA* mRNA by extending an oligonucleotide (HP859) complementary to sequences within the *rnjA* ORF. A major reverse transcriptase stop was observed at position −254 with respect to the *rnjA* start codon (Fig. 1, lane 5). In order to verify that this signal represents a genuine 5′ end, we performed an S1 nuclease analysis using the same oligonucleotide (HP859) to define the 5′ end of the S1 probe. Detection of a signal of the same size as the reverse transcriptase product (Fig. 1, lane 7) confirmed the position of the *rnjA* mRNA 5′ end. Located upstream of the *rnjA* gene is the gene *ykzG* encoding a 69-amino-acid (aa) protein belonging to the family UPF0356 of uncharacterized proteins (UniProt), which comprises polypeptides of about 70 residues in length which occur in *Bacillales* and *Lactobacillales*.

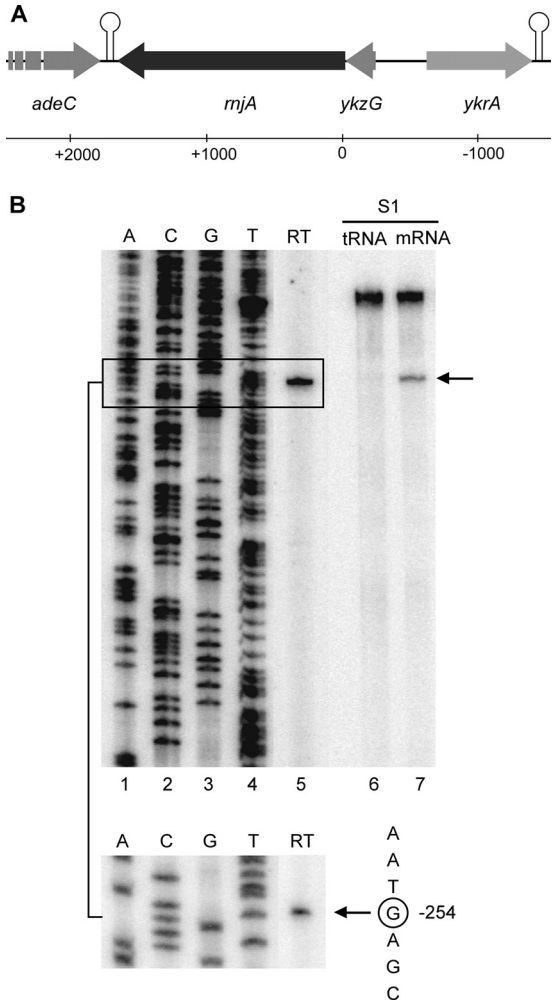

FIG 1 Determination of the transcription initiation point of the *rnjA* mRNA. (A) Schematic diagram of the *rnjA* locus. The stem-loop structures represent factor-independent transcription terminators which are predicted to function in both directions. The *adeC* gene encodes an adenine deaminase. Numbering is in base pairs. (B) Primer extension and nuclease S1 analysis. Primer HP859 was hybridized to total RNA from a wild-type strain and extended by reverse transcriptase (lane RT). The exact position of the major signal was determined on a high-resolution gel and is indicated by an arrow in the lower panel. The G residue at position −254 with respect to the *rnjA* start codon that corresponds to the reverse transcriptase stop is circled (lower panel). S1 nuclease analysis using the same primer (HP859) to create the 5′ end of the probe is shown in the upper panel, lanes 6 and 7. The S1 probe was hybridized to tRNA (control, lane 6) or total mRNA isolated from a *B. subtilis* wild-type strain (lane 7). The major signal observed is indicated by an arrow.

The mRNA encoding RNase J1 is likely synthesized as a bicistronic transcript including the upstream *ykzG* ORF (Fig. 2). Immediately upstream of the *ykzG-rnjA* mRNA 5′ end, we found a perfect sigma A-type −10 box but no strong −35 promoter consensus element. In order to exclude the possibility that the major promoter resided further upstream and that the identified mRNA 5′ end was due to a processing event, we constructed two transcriptional *lacZ* fusions containing untranslated regions upstream