Soj Antagonizes Spo0A Activation of Transcription in *Bacillus subtilis*

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The ParA family protein Soj appears to negatively regulate sporulation in *Bacillus subtilis* by inhibiting transcription from promoters that are activated by phosphorylated Spo0A. We tested in vitro Soj inhibition of Spo0A-independent variants of a promoter that Soj inhibited (*PspoIIG*). Transcription from the variants was less sensitive to Soj inhibition, suggesting that inhibition of wild-type *PspoIIG* was linked to transcription activation by Spo0A.

Bacillus subtilis responds to nutrient deprivation and high population densities by initiating a pattern of morphological development resulting in the release of a dormant endospore (9, 10, 12, 26). The decision to sporulate is governed by the levels of phosphorylated Spo0A (Spo0A~P) (9, 10, 12, 26). One central function of Spo0A~P is to activate the transcription of essential stage II operons, including *spoIIA*, *spoIIE*, and *spoIIG* (2, 9, 10, 24, 26–28). The *spoIIA* and *spoIIG* operons encode sporulation-specific sigma factors that direct early developmental transcription in the forespore and mother cell compartments, respectively (11, 15, 16, 19).

The *spo0J* operon is a dicistronic unit encoding proteins that belong to the ParA and ParB families, whose members are widely dispersed throughout the bacteria (1, 7, 17). Spo0J is a sequence-specific DNA binding protein in the ParB family that binds in vitro and in vivo to several origin-proximal DNA sites, termed *parS* (18). Deletion of *spo0J* results in a phenotype with a low frequency of anucleate cells accumulating during growth and a block to sporulation before stage II (14, 21). The sporulation block is relieved by the deletion of the *parA* homologue, *soj* (14). Transcription from *spoIIA*, *spoIIE*, and *spoIIG* promoters is severely reduced in a $\Delta spo0J$ mutant and restored close to wild-type levels in a $\Delta soj spo0J$ double mutant (14, 22). Soj associates with the *spo0A*, *spoIIA*, *spoIIE*, and *spoIIG* promoters in vivo, and these associations are more pronounced in the absence of Spo0J (22, 23). In vitro Soj inhibits transcription from the *spoIIG* promoter (*PspoIIG*) activated by either Spo0A~P or the constitutively active C-terminal domain of Spo0A (Spo0AC) (3). These results imply that Soj acts as a negative regulator of sporulation by inhibiting transcription that is activated by phosphorylated Spo0A.

In vitro, Soj dissociates complexes formed by incubating a DNA fragment containing *PspoIIG*, Spo0AC, and RNA polymerase (RNAP) (3). The Soj-induced dissociation of the promoter-Spo0A-RNAP is unusual, and it was suggested that Soj might act at a step in initiation that follows the action of Spo0A (3). To further explore the mechanism of Soj action, we first

needed to distinguish whether or not Soj specifically blocked an activity of Spo0A. We reasoned that if Soj targeted a step in transcription that was independent of Spo0A, then Soj inhibition of a *PspoIIG* variant that had been mutated so it no longer required Spo0A for high activity would be the same as its inhibition of wild-type *PspoIIG*. To test this idea, we constructed a set of mutated *spoIIG* promoters affected only in the spacer between the –10 and –35 consensus sequences. As reported here, in vitro transcription from these spacer-shortened *spoIIG* promoters was independent of Spo0A and showed an altered response to Soj.

Characterization of the shortened *spoIIG* promoters. Bases between the –10 and –35 consensus sequences of *PspoIIGBglII* were deleted to create the shortened promoters used in this study (Fig. 1A). Promoters with deletions of 6, 5, and 4 bp were termed *PspoIIG16*, *PspoIIG17*, and *PspoIIG18*, where the numbers indicate the length of the spacer (in bases) between the –10 and –35 sequences. The –10 and –35 consensus sequences as well as the Spo0A binding sites (“0A boxes”) of *PspoIIG* were not affected by the deletions.

The transcription initiation requirements of the shortened promoters were tested and compared to those of wild-type *PspoIIG* by in vitro transcription assays (Fig. 1B), as described in detail elsewhere (3). For all templates, addition of both ATP and GTP along with DNA and purified *B. subtilis* RNAP (25), before the addition of heparin, was necessary for high levels of transcription. Inclusion of CTP with the incubation of RNAP plus ATP and GTP had little effect on initiation, except at *PspoIIG17*, where it markedly increased transcription initiation. Primer extension experiments indicated that the transcription start site for all three shortened promoters was the same as that for *PspoIIG* (data not shown). We concluded that the deletion of the bases between the –10 and –35 sequences did not change the nucleotide dependence of initiation or the transcription start site of the shortened promoters compared to that of *PspoIIG*.

While Spo0A~P or Spo0AC was required for high-level transcription from *PspoIIG*, this was not true for *PspoIIG17* or *PspoIIG18*; in fact, inclusion of Spo0AC in transcription assay reactions with *PspoIIG17* or *PspoIIG18* template DNA led to 22 and 23% decreases in transcription, respectively (Fig. 1C). This provides the first direct evidence that the action of

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FIG. 1. (A) Sequence of *PspoIIIG* and shortened *spoIIIG* promoters. The region between +1 and -60 is indicated. The -10 and -35 sequences are in boldface letters. Underlined bases indicate 0A boxes. Dashes indicate bases deleted from BglIII-treated pUCIIBglIII*trpA* by incubation with mung bean nuclease followed by ligation. Italic letters in *PspoIIIGBglIII* indicate a BglIII site that was introduced into pUCIIBglIII*trpA* (24). (B) Nucleotide initiation requirements for the indicated promoter. The first addition indicates the initiation nucleotides incubated with promoter DNA (6 nM) and RNAP (25 nM) (and Spo0AC at 1 μ M when *PspoIIIG* was used) for 2 min at 37°C. The second addition indicates elongation nucleotides incubated for an additional 5 min in the presence of heparin (15 μ g ml⁻¹) in a 20- μ l reaction mixture. Transcription was monitored by [α -³²P]GTP (7.7 μ M, 23 Ci mmol⁻¹) incorporation; all other nucleotides were present at 0.6 mM. Values in parentheses indicate the amount of transcript, measured relative to the amount of transcript from *PspoIIIG18* with ATP, GTP, and CTP as the initiating nucleotides. (C) Transcription from *PspoIIIG* and shortened promoters in the presence and absence of Spo0AC with ATP and GTP as the initiating nucleotides. Products from transcription assays were separated by electrophoresis on an 8% denaturing polyacrylamide gel. The major transcript was detected and quantified by exposure of the gel to a phosphor screen followed by analysis with Imagequant software (v5.0; Amersham Biosciences). Error bars represent the standard deviation from the average of four independent transcription assay reactions.

Spo0AC in stimulating *PspoIIIG* is to overcome the barrier to transcription imposed by the 22-bp spacer, since reducing the spacer eliminates the Spo0AC requirement.

Analysis of ATP binding and hydrolysis by purified Soj. We measured the ATP binding and hydrolysis by Soj to assess whether the purified protein was active. Soj was purified as previously described (3), except that transcription reaction buffer (25) with 15% glycerol, 300 mM sodium chloride, and 100 μ M ATP, but without either dithiothreitol or bovine serum albumin, was used as the column buffer. This change increased Soj yield and solubility. Soj was dialyzed against storage buffer (same as described above, except with 50 mM sodium chloride) and stored at -70°C.

Soj (5 μ M) was incubated with 50 μ M nucleotide for 2 min at 37°C, UV cross-linked on ice, and precipitated essentially as described previously (6). The precipitate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-

PAGE), and the dried gel was exposed to a phosphor screen and visualized with a PhosphorImager PSI (Amersham Biosciences). A major band at 28 kDa was present, corresponding to Soj cross-linked to radionucleotide when incubated with ATP (Fig. 2A). No cross-linked species appeared if the sample was not UV treated. Soj cross-linked dATP to a lesser extent but did not cross-link to GTP (Fig. 2A).

Soj-mediated hydrolysis of ATP, detected by thin-layer chromatography (6), was linear for the first 16 min of the assay (Fig. 2B). The rate of ATP hydrolysis increased up to approximately 140 μ M ATP (Fig. 2C), and a turnover number of $(4.9 \pm 1.8) \times 10^{-3} \text{ s}^{-1}$ was calculated. The ATP turnover of Soj was slightly lower than that of ParA from *Caulobacter crescentus* (2.6-fold) (8) and higher than those of *Escherichia coli* P1 plasmid ParA and MinD (4- and 10-fold, respectively) (4-6, 13). This demonstrates that Soj, like other ParA family proteins, is a weak ATPase. The ATPase activity of ParA from the

FIG. 2. ATP binding and hydrolysis activities of Soj. (A) Nucleotide binding specificity of Soj. α^{32} -P-labeled nucleotides ($50 \mu\text{M}$, $24.8 \text{ Ci mmol}^{-1}$) were incubated with $12.5 \mu\text{M}$ Soj or 0.1 mg of bovine serum albumin (BSA) ml^{-1} and UV cross-linked followed by trichloroacetic acid precipitation and solubilization in SDS-PAGE sample buffer as described previously (6). Samples were separated by SDS-PAGE on a 12% polyacrylamide gel, and the dried gel was exposed to a phosphor screen. An arrow indicates the position of Soj as determined by staining of the gel with Coomassie brilliant blue prior to drying. (B) SDS-PAGE (12% polyacrylamide) of purified Soj ($4.6 \mu\text{g}$), judged to be over 99% pure by staining with Coomassie brilliant blue, and low-range molecular weight standards (Bio-Rad Laboratories). (C) Soj time course of ATPase activity. Soj ($4.2 \mu\text{M}$) and $100 \mu\text{M}$ [α^{32} P]ATP (9.9 Ci mmol^{-1}) were incubated at 37°C , and samples were applied as spots onto a polyethyleneimine thin-layer chromatography plate and developed as described previously (6). (D) ATP dependence of ATPase. Soj ($1.8 \mu\text{M}$) was incubated with the indicated [α^{32} P]ATP

P1 plasmid of *E. coli* is stimulated 10-fold in the presence of ParB and DNA (4). The ATPase activity of MinD is stimulated 10-fold by the presence of MinE and phospholipids (13). However, we have not been able to detect stimulation of Soj ATPase by simple addition of Spo0J and/or DNA.

Inhibition of transcription from shortened *pspIIG* promoters. Transcription from Spo0AC-activated *PspIIG* was inhibited 10-fold relative to transcription in the presence of Soj storage buffer alone over the range of ~ 0.15 to $3.5 \mu\text{M}$ Soj (Fig. 3A). Inhibition by $\sim 0.15 \mu\text{M}$ Soj is more obvious in the insert in Fig. 3A, where Soj concentrations are plotted on a logarithmic scale. Changing the input of Spo0AC did not appreciably alter the Soj inhibition curves, suggesting that Soj and Spo0A do not compete for binding to RNAP (data not shown). Two promoters not expected to be affected by Soj were also tested. The *A2* promoter (*P42*) is from bacteriophage $\phi 29$ and was previously reported to be unaffected by Soj in vitro (3). The *abrB* gene promoter (*PabrB*) was reported to be unaffected by mutational loss of the *spo0J* gene in vivo (3). Levels of transcription inhibition from *P42* and *PabrB* were similar, occurring over a broad range and beginning to decrease only when Soj concentrations approached $1 \mu\text{M}$ (Fig. 3B and C). These data indicated that Soj has a low-affinity inhibitory property seen at *P42* and *PabrB* and a high-affinity inhibitory property seen at *PspIIG*.

Levels of Soj inhibition of transcription from *PspIIG16*, *PspIIG17*, and *PspIIG18* showed similar profiles, and only the data for inhibition of *PspIIG18* are shown (Fig. 3D). Soj-mediated transcription inhibition of *PspIIG18* was similar to that of *P42*, producing similar inhibition curves over the same Soj values. The sensitivity of *PspIIG18* to Soj inhibition was not affected by the presence of Spo0AC. These results indicated that Soj transcription inhibition at *PspIIG18* was of the low-affinity type. Thus, loss of the requirement for Spo0AC activation changed Soj-dependent inhibition of transcription from *PspIIG* from the high-affinity type to the low-affinity type.

A previous study concluded that Soj did not inhibit transcription from *P42* (3). The data in that paper showed a 30% reduction of transcription at $\sim 4.5 \mu\text{M}$ Soj, which is similar to the inhibition level shown in Fig. 3B. The reduction was previously interpreted as being not due to Soj. The experiments reported here carefully controlled reaction conditions with respect to ionic strength and organic solvents and used higher concentrations of Soj. These experiments allowed us to resolve the low-affinity inhibition effect at *P42*, *PabrB*, and the shortened promoters.

Because *PspIIG* transcription is dependent on activated Spo0A, we were previously unable to tell whether Soj inhibition reflected interaction of Soj with a DNA sequence at *PspIIG* or an interaction with the RNAP-Spo0A complex. The data reported here indicate that the high-affinity inhibition reflects the latter, since it was seen only with the Spo0A-activated promoter. If the low-affinity inhibition results from

concentrations (12.4 to $0.62 \text{ Ci mmol}^{-1}$) for 16 min at 37°C , and the products were analyzed by exposure of the developed thin-layer chromatography plates to a phosphor screen.

FIG. 3. Soj-mediated transcription inhibition. The indicated concentrations of Soj were incubated with *PspIIIG* (the insert indicates transcription at lower Soj concentrations) (A) or *PA2* (B) and ATP plus GTP (and Spo0AC at 1 μ M when *PspIIIG* was used) for 2 min at 37°C before the addition of RNAP. After another 2 min, heparin and nucleotides required for transcript elongation were added, and the reaction was stopped after 5 min of elongation. The relative transcription was measured as the percentage of transcription in the absence of Soj. To maintain a consistent ionic strength among reaction samples, Soj was diluted into storage buffer and a constant volume was added to each reaction. The same volume of storage buffer alone was added to the control reactions. (C) Soj-mediated transcription inhibition at *PabrB*. Transcription assays were performed as described for panel A, except the initiation nucleotides used were ATP plus GTP plus UTP, and the transcripts were separated by electrophoresis on a 12% denaturing polyacrylamide gel. (D) Soj-mediated transcription inhibition at *PspIIIG18* in the presence (◆) or absence (■) of 1 μ M Spo0AC. The assays were performed and analyzed as described for panel A. Error bars represent the standard deviation from the average percentage of at least three independent transcription assays.

interaction of Soj with a specific DNA sequence, it must be degenerate, since comparison of the *PA2*, *ParbB*, and *PspIIIG* sequences did not reveal any obvious common motifs. Given the low ATPase activity of Soj, it is unlikely that the low-affinity inhibition reflects loss of ATP during the transcription reaction. Phosphorylated Spo0A activates approximately 40 genes, the majority of which encode sporulation-specific proteins (20). Potentially most of these will also be negatively regulated by Soj.

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