The SpoOA protein of *Bacillus subtilis* is a repressor of the *abrB* gene

(footprinting/transcription/sporulation)

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ABSTRACT The *spoOA* gene of *Bacillus subtilis* is critical for the initial stages in the developmental cycle leading to the formation of an endospore. We show that one function of the SpoOA protein is to negatively regulate another regulatory locus, *abrB*, which controls the expression of many genes associated with the onset of sporulation. Purified SpoOA protein binds to a specific region of the *abrB* promoter and functions as a repressor of transcription in an *in vitro* assay. The binding of the SpoOA protein is independent of the binding of the AbrB protein, which is known to autoregulate its expression. This independence mirrors the temporal sequence of events in *abrB* control.

The products of the *Bacillus subtilis* *spoO* genes are required for the initiation of sporulation in this organism (1, 2). Mutations of *spoO* in *B. subtilis* block sporulation at its earliest stage and exhibit pleiotropic effects on the expression of a wide variety of gene products associated with the end of exponential growth and the onset of sporulation: the transition stage (1, 3). The most pleiotropic of the *spoO* mutations occur at the *spoOA* locus.

It is believed that SpoOA is the main component through which the environmental signals for sporulation are relayed to the transcriptional machinery of the cell (4, 5). The *spoOA* gene codes for a protein of 29,700 Da (6), which exhibits amino acid sequence homology with the regulator class of molecule (e.g., OmpR, NtrC, CheB, CheY, etc.) in prokaryotic two-component regulatory systems (7–10). The SpoOA protein regulates the onset of stationary phase and sporulation, in part, by controlling the expression of the *abrB* gene. The product of the *abrB* gene is a repressor that functions to prevent the expression of transition stage-specific genes during vegetative growth (11, 12). Among the genes regulated by *abrB* are some that are necessary for normal sporulation: *spoOE* (5), *spoOH* (13), and *spoVG* (14). During the transition stage, production of the AbrB protein decreases, thus allowing for the derepression of promoters regulated by AbrB (11). The modulation of AbrB synthesis depends upon the SpoOA protein (11, 15).

Although a large body of genetic evidence implicated the SpoOA protein in regulatory phenomena occurring at the onset of sporulation, the actual biochemical function of the SpoOA protein was unknown. In this communication we show that the SpoOA protein binds to a specific region of the *abrB* gene located downstream from the transcriptional start sites and functions as a repressor of transcription *in vitro*.

MATERIALS AND METHODS

DNase and Methylation Protection Assays. The 800-base-pair (bp) *Bam*HI–*Hind*III fragment of pJM5134 (11)

containing the *abrB* promoter and upstream region was labeled at the *Bam*HI end by using either [α -³²P]dATP (3000 Ci/mmol, Amersham; 1 Ci = 37 GBq) and the Klenow fragment of DNA polymerase I (Bethesda Research Laboratories) or [γ -³²P]ATP (7000 Ci/mmol, ICN) and T4 polynucleotide kinase (United States Biochemical) and isolated by PAGE. The DNase I protection assays (footprinting) were done as described (12) with labeled DNA at $\approx 1\text{--}3 \times 10^{-9}$ M final concentration and highly purified SpoOA protein (see below). The method used for determining which purine (both adenine and guanine) residues were protected from methylation by dimethyl sulfate due to binding of SpoOA was as follows. Approximately 8×10^4 cpm (1×10^{-14} mol) of labeled *abrB* promoter DNA and 6 μ g of sheared salmon sperm DNA were incubated with 10 μ g (3×10^{-10} mol) of SpoOA protein for 10 min at 37°C in a 20- μ l volume containing 0.04 M Hepes (pH 8), 0.1 M KCl, 0.01 M MgCl₂, bovine serum albumin at 0.1 mg/ml, 0.1 mM dithiothreitol, 0.1 mM Na₂EDTA, and 10% (vol/vol) glycerol. The reaction was allowed to cool to room temperature (2 min), and 280 μ l of the G buffer supplied with the New England Nuclear Maxam and Gilbert sequencing kit and 1 μ l of dimethyl sulfate were added and incubated on ice for 10 min. The reaction was stopped, and the DNA was precipitated three times with ethanol (16). The DNA was resuspended in 20 μ l of H₂O chilled to 0°C, and 5 μ l of 0.5 M HCl was added and incubated for 2 hr at 0°C with occasional mixing. At the end of this time 200 μ l of 0.3 M sodium acetate and 750 μ l of ethanol were added, the DNA was collected by centrifugation for 10 min in a microcentrifuge, and the DNA was washed with 70% ethanol and dried. The DNA was then dissolved in 10 μ l of 0.1 M NaOH/1 mM EDTA and heated to 90°C for 30 min. The mixture was lyophilized and resuspended in gel loading dye [0.1% xylene cyanol/0.1% bromophenol blue/10 mM disodium EDTA/95% (vol/vol) deionized formamide]. As the unprotected control, the procedure was carried out as above but without SpoOA protein. Identical amounts (in cpm) of protected and unprotected reactions were electrophoresed alongside each other on 6% polyacrylamide/6 M urea sequencing gels.

Methylation protection experiments for guanine residues only were performed as described (12).

In Vitro Transcription Assays. RNA polymerase activity at the *abrB* promoters was assayed by using a modification of the single-round transcription assay (17). Briefly, pJM5134 DNA (11), linearized at the *Eco*RI site downstream from the promoter region, was incubated in 40 mM Tris (pH 7.9)/20 mM MgCl₂ with GTP, CTP, and UTP (400 μ M final concentration) at 37°C for 2 min. Aliquots (11.3 μ l, constant volume) of SpoOA protein (diluted in 0.025 M Tris, pH 7.9/0.1 mM EDTA/1 mM MgCl₂/0.01 M KCl/1 mM CaCl₂/7 mM 2-mercaptoethanol/30% glycerol containing phenylmethylsulfonyl fluoride at 50 μ g/ml) were added to the indicated final concentrations and incubated for 1 min to allow SpoOA binding. After 1-min incubation, RNA polymerase was added to the 78 nM (final concentration) and incubated for 2 min, at

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which time heparin (final concentration of 5 $\mu\text{g}/\text{ml}$) and 8.2 μCi of [α - ^{32}P]ATP (Amersham) at 10 μM (final concentration) were added. The heparin-resistant complexes were allowed to elongate for 9 min. Reactions were stopped by the addition of 10 μl of 10 M urea/0.2 M Tris base/0.2 M boric acid/4 mM EDTA to give a final volume of 50 μl . Aliquots (20 μl) were electrophoresed through a 10% polyacrylamide gel containing 7 M urea at 600 V for 2 hr. Regions of the gel containing specific transcripts were localized by autoradiography and excised; the amount of incorporated label was determined by measuring the Cerenkov radiation in each slice. The 5' ends of the transcripts were determined by eluting the RNA from gel slices and using the transcripts in S1 mapping experiments as described (17). Inhibition reactions containing different combinations of nucleoside triphosphates were examined. As has been reported for other *Bacillus* promoters (17), elongation from the P2 promoter of *abrB* in the presence of heparin (used to eliminate second-round transcription) required prior incubation with the initiating nucleotides. Inhibition experiments containing GTP and UTP alone (without CTP) produced identical data to that seen in Fig. 4. No set of nucleotides in the absence or presence of heparin produced a significant level of a transcript that mapped to the P1 site.

Purification of SpoOA Protein. The SpoOA protein was purified to homogeneity from an *Escherichia coli* strain overproducing this protein. A detailed report of the purification scheme is in preparation, but the purity of the protein prepared in this manner has been demonstrated (18).

RESULTS

Location of SpoOA Binding on *abrB* DNA. Ikeuchi *et al.* (19) showed that purified SpoOA protein possessed nonspecific DNA-binding activity as well as the ability to preferentially

FIG. 1. DNase I protection experiments. Results obtained when the nontemplate strand is labeled at its 3' end. Lanes: 1, 5 μM SpoOA protein; 2, 3.5 μM SpoOA; 3, 2.5 μM SpoOA; 4 and 5, no protein. The Maxam and Gilbert A + G (Pu) and C + T (Py) reactions are shown for reference; numbering is relative to the start point of P2 transcription.

FIG. 2. SpoOA binding to the *abrB* promoter region. Bases protected from DNase I cleavage due to SpoOA binding are indicated for both strands by uppercase letters. Purines showing reduced methylation with SpoOA are indicated by an asterisk (*), and those showing enhanced methylation are indicated by a caret (^). The putative SpoOA recognition sequences TGNCGAA are bracketed. Start point of transcription for the P2 promoter is shown for reference, and the bases are numbered relative to it. (The P1 promoter start point is at -14 by this convention.)

bind a specific restriction fragment of $\phi 105$ DNA. Evidence that the SpoOA protein bound specifically to a chromosomal DNA fragment containing the *abrB* promoter region was first obtained in gel mobility-shift experiments (data not shown). To define the precise location of binding, DNase I protection assays (footprinting) were performed. Fig. 1 shows a representative footprint when the nontemplate strand was labeled, and Fig. 2 illustrates the deduced location of the protected area on this and on the template strand in relation to the known *abrB* promoter elements. The SpoOA protein binds to and affords protection to sequences lying downstream of the transcription start points of the two *abrB* promoters: +7 to +34 (relative to P2) on the nontemplate strand and +5 to +32 (relative to P2) on the template strand. This location of SpoOA binding does not overlap with the region of AbrB binding that extends upstream from the -15 position of P2 promoter (12, 15).

SpoOA-Purine Contacts. To define more precisely which sequences in the footprint region are most likely to be involved in SpoOA binding as judged by close contact between the protein and DNA we performed methylation protection experiments on the *AbrB* promoter. Fig. 3 shows

FIG. 3. Dimethyl sulfate protection experiment. Results obtained when the nontemplate strand of the *abrB* promoter was labeled at its 3' end. The extent of dimethyl sulfate modification of purine residues, as judged by subsequent susceptibility to cleavage, with and without SpoOA protein. Numbering system is relative to the P2 start point.

the results of such an experiment, and Fig. 2 illustrates these results in a schematic manner. Under conditions when both the adenines and guanines are subject to methylation and subsequent chain cleavage, binding of the SpoOA protein afforded protection to the following bases (all numbers relative to the P2 start point): guanine at +12, guanine at +22, adenine at +23 on the nontemplate strand; guanine at +14, guanine at +24 on the template strand. Somewhat weaker protection was detected at the adenine at +20 on the nontemplate strand. Enhanced methylation was seen for the guanine at +15, adenine at +19, and guanine at +25 on the nontemplate strand. When conditions were such that only the methylation of the guanines was examined, all the above-mentioned patterns were seen, but, in addition, in one experiment the guanine at +28 also appeared to be slightly protected. Examination of the sites of close SpoOA contact reveal what may be a consensus binding sequence: TG(A or T)CGAA. As seen in Fig. 2, this sequence occurs twice in the footprint region (separated by 3 bp), and all the protected purines are in one or the other copy of the sequence (except for the weakly protected adenine at +20 described above). In addition, the first thymine of the first sequence at +11 is approximately one turn of the helix (10 bp) away from the first thymine of the second sequence at +21.

SpoOA Effect Upon *in Vitro* *abrB* Transcription. Previous studies had shown that the *spoOA* gene was involved in transcriptional regulation of the P2 promoter of the *abrB* gene but had no apparent effect upon the P1 promoter *in vivo* (11). The fact that the SpoOA protein bound downstream of the P2 start site *in vitro* suggested that the SpoOA protein might directly affect the transcription from P2. To examine this point we performed *in vitro* transcription assays on linearized *abrB* template DNA by using plasmid pJM5134. Under the conditions used, more than one transcription product was seen. The different RNAs were separated by electrophoresis and eluted from the acrylamide gel; the 5' end of each was determined by S1 mapping. One RNA that accounted for >75% of the total transcription products was identified by S1 nuclease mapping as initiating at the same site as the P2 promoter initiates *in vivo* (11). The effect of SpoOA on the synthesis of this RNA was determined. When increased concentrations of SpoOA protein were added to transcription reactions before addition of RNA polymerase (Fig. 4), tran-

FIG. 4. Effect of the SpoOA protein upon *in vitro* transcription of the P2 promoter. RNA polymerase-directed incorporation of [α - 32 P]ATP into the P2 transcript *in vitro* is plotted as a function of SpoOA concentration.

scription activity from the P2 promoter was exponentially inhibited. Half-maximal inhibition occurred at 1.5 μ M SpoOA protein. The level of transcription from the P1 promoter was too low to determine accurately. The 5' ends of the minor RNAs observed from transcription of linearized pJM5134 indicated transcription initiation start sites downstream from the P2 promoter. Synthesis of all these transcripts was inhibited by prior incubation with SpoOA. As *in vivo* data did not show additional promoter sites for *abrB* (11), we believe that these sites represent a feature of the *in vitro* system. We examined the effect of the SpoOA protein on *in vitro* transcription from the P1 promoter of the *rrnB* operon of *B. subtilis*. A sequence search of this promoter showed that it did not contain the SpoOA binding sequence. Prior addition of SpoOA protein to the *rrnB* P1 promoter had no effect on transcription up to 5 μ M SpoOA (data not shown).

Independence of SpoOA and AbrB Binding to *abrB* DNA.

The only known regulators of the *abrB* gene are the SpoOA protein (11) and the AbrB protein itself (11, 12, 15). Because the site of SpoOA binding, as judged by footprinting, does not overlap the site of AbrB protein binding (12), it was of interest to determine whether the two proteins bound independently of each other or whether binding of one influenced behavior of the other. To examine this question we performed footprinting assays with both proteins: either binding SpoOA first, binding AbrB first, or binding both simultaneously. We

FIG. 5. Independence of SpoOA and AbrB binding to the *abrB* promoter as judged by DNase I protection experiments. Results obtained when the template strand is labeled at its 5' end. Lanes: 1, 1 μ M SpoOA and 2 μ M AbrB proteins; 2, 0.5 μ M SpoOA and 2 μ M AbrB; 3, 2 μ M AbrB; 4, 0.5 μ M SpoOA; 5 and 6, no protein. For lanes 1 and 2, SpoOA protein was added to the DNA and allowed to incubate for 2.5 min at 37°C; then AbrB protein was added, and incubation was continued for 2.5 min before the complexes were subject to DNase I treatment. Entirely identical results were obtained in experiments (data not shown) in which AbrB protein was added first. The Maxam and Gilbert sequencing reactions are shown for reference; numbering system is relative to the start point of P2 transcription.

did not observe any type of effect of one protein binding upon the binding of the other (Fig. 5). For example, a characteristic hypercleavage site at the upstream edge of the footprint region (position +4, Fig. 5) that is induced by the binding of SpoOA is not visibly affected by the presence of AbrB protein (Fig. 5, lanes 1 and 2), even though the AbrB protein protects sequences beginning just 17 bp further upstream (endpoint at -14). Thus, the apparent conformational change caused by SpoOA binding that results in the hypercleavage site is apparently not altered by the binding of AbrB, further implying independence of binding.

DISCUSSION

In this communication we show that SpoOA can bind specifically to the DNA of one of its targets, *abrB*, and repress its transcription. This demonstrates biochemical activity for the SpoOA protein that can be correlated to events occurring during the earliest stages of sporulation and another biochemical activity for an *spoO*-encoded product; the *spoOH* gene product has been reported to be an alternate sigma factor (20).

Earlier genetic results have implicated SpoOA in the control of *abrB* expression. Mutations of *spoOA*, in addition to their effect on sporulation, cause the lack of expression of certain genes, such as subtilisin (*aprE*), associated with the earliest stages of sporulation (1). Second-site mutations in the *abrB* gene suppressed this phenotype, although these mutations did not restore sporulation (21–24). From this genetic evidence it was hypothesized that the *abrB* product functioned as a repressor of certain transition-stage genes and that, upon commencement of sporulation, the SpoOA protein repressed the *abrB* gene; the latter, in turn, led to expression of the *abrB*-repressible subset of genes (11). This scheme received further support when the AbrB protein was shown to be bound to the promoter regions of its targets (12) and, in at least one case, to repress *in vitro* transcription (unpublished observations). With our results showing the ability of SpoOA to repress *abrB* transcription, many of the major features of the initial hypothesis have now been proven.

The SpoOA protein plays a central role in the onset of sporulation in *B. subtilis* (4). It appears to be the pivotal component through which the signal for sporulation is relayed to the regulatory circuits of the vegetative cell. Although the nature of the environmental signal for spore formation is not known (25, 26), the signal probably triggers a modification of the SpoOA protein leading to altered regulatory properties, which, in turn, begin the developmental sequence leading to spore production. The nature of the modification has recently been shown to involve a reversible phosphorylation of the SpoOA protein (K. Trach, D. Burbulys, and J.A.H., unpublished observations; ref. 18); this is a not-too-surprising result because SpoOA is known to show extensive amino acid homology with the regulator class of molecule in the prokaryotic two-component regulatory systems (8–10), many of which have also been shown to be subject to phosphorylation control. By further analogy to the two-component regulator class proteins, such as OmpR and NtrC, the phosphorylation is believed to change the interaction of SpoOA with either the transcriptional machinery of the cell or directly with the promoter DNA of various target genes. Whether phosphorylation is required for repression of the *abrB* gene by SpoOA *in vivo* or whether this activity of the SpoOA protein is independent of phosphorylation is unknown. However, studies in kinase mutants suggest that phosphorylation of SpoOA is not required for its activity on the *abrB* promoter (K. Trach and J.A.H., unpublished work; ref. 18). Thus phosphorylation of the SpoOA protein most likely potentiates its role in the control of functions other than *abrB* repression. If repression of *abrB* were independent of

the phosphorylated state of SpoOA, the question arises as to the mechanism of activation of SpoOA repression of *abrB*. Because SpoOA synthesis has been shown to increase ≈ 2 -fold at the end of vegetative growth (27), this increased level of SpoOA protein could be sufficient to repress *abrB*. We believe it is equally feasible that an effector molecule is produced that activates SpoOA activity toward *abrB*.

Control of *abrB* transcription cannot be the sole function of SpoOA: *abrB* mutations do not correct the sporulation defect found in *spoOA* mutant strains. Therefore, SpoOA must be involved in the control of other genes necessary for sporulation. Possibly this action includes not only negative control of other repressors such as AbrB but also could involve positive activation of certain genes.

The *abrB* gene is transcribed from dual promoters: an upstream promoter (P1) and a downstream promoter (P2) that is regulated by SpoOA (11). During vegetative growth the major regulation of AbrB is apparently a form of autoregulation (15). However, with the beginning of the transition state and onset of sporulation, the SpoOA protein becomes the major repressor of *abrB* transcription (11). That the binding sites for the AbrB and SpoOA proteins are distinct and do not apparently interact (Fig. 5) is consistent with the different temporal roles that the repressors assume in *abrB* regulation. The upstream endpoint of SpoOA binding, as determined by DNase I footprinting, is located 16–19 bp downstream from the P1 start point and 2–5 bp downstream from the P2 start point (Fig. 2). Thus, although bound SpoOA may sterically inhibit RNA polymerase initiation at P2, it seems unlikely that bound SpoOA could do so at P1, especially because we have shown that SpoOA binding in no way affects the binding of another protein (AbrB) in this region (Fig. 5). Bound SpoOA may be able to block the progression of RNA polymerase initiated at P1 or, alternatively, SpoOA binding may disrupt some type of secondary structure at P1 necessary for RNA polymerase, but not AbrB, binding.

Examination of the sequences to which SpoOA binds reveals a putative recognition site (Fig. 2). The occurrence of two sequences of the type TG(A/T)CGAA spaced one turn of the helix apart and in which all of the apparent close purine–protein contacts occur argues that this is the SpoOA-binding site. And, in fact, sequences of the type TGNCGAA have been located in the promoter regions of other genes known to be controlled by SpoOA: *spoOA* itself (6), *spoOF* (28), *spoIIA* (29), and $\phi 29$ pE³ (30). However, in all these cases there is only a single element of the sequence; none show a repeat of the element one helix turn away. Thus binding of SpoOA at these sites may be intrinsically different, and there may be other contextual elements that determine the precise nature of the binding sites.

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