Promoter Activation by Repositioning of RNA Polymerase[▽]

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Received 18 January 2008/Accepted 13 February 2008

Spo0A, a classical two-component-type response regulator in *Bacillus subtilis*, binds to a specific DNA sequence found in many promoters to repress or activate the transcription of over 100 genes. On the *spoIIG* promoter, one of the Spo0A binding sites, centered at position –40, overlaps a consensus –35 element that may also interact with region 4 of the sigma A (σ^A) subunit of RNA polymerase. Molecular modeling corroborated by genetic evidence led us to propose that the binding of Spo0A to this site repositions σ^A region 4 on the promoter. Therefore, we used a chemical nuclease, *p*-bromoacetamidobenzyl-EDTA-Fe, that was covalently tethered to a single cysteine in region 4 of σ^A to map the position of σ^A on the promoter. The results indicated that in the absence of Spo0A, σ^A region 4 of the RNA polymerase was located near the –35 element sequence centered at position –40. However, in the presence of Spo0A, σ^A region 4 was displaced downstream from the –35 element by 4 bp. These and other results support the model in which the binding of Spo0A to the *spoIIG* promoter stimulates promoter utilization by repositioning prebound RNA polymerase and stabilizing the repositioned RNA polymerase-promoter complex at a new position that aligns σ^A region 2 with the –10 region sequences of the promoter, thus facilitating open complex formation.

Bacterial RNA polymerase, a multisubunit enzyme, recognizes and binds to promoters by interacting with specific sequences of DNA located in several regions of the promoter (reviewed in references 8 and 52). Regions 2 and 4 of the σ^{70} -type sigma subunit of RNA polymerase interact with sequences of the promoter centered about 10 bp (–10 element) and 35 bp (–35 element), respectively, upstream from the start point of transcription (12, 19, 33, 49, 53). In addition to the primary sigma factor, most bacteria contain secondary sigma factors that recognize and bind to different sequences at the –10 and –35 regions of their cognate promoters or, for some sigma factors, at other regions of their cognate promoters (reviewed in reference 22). Region 3 of the sigma factor interacts with sequences immediately upstream from the –10 region, the extended –10 region, in some promoters (33, 43). In addition, the carboxyl-terminal domain of the alpha subunit of RNA polymerase binds in some promoters to a 20-bp sequence (the UP element) located upstream from the –35 element (40). Promoters usually have only a subset of these four recognition elements. In many cases, promoters containing suboptimal RNA polymerase binding elements require an additional factor, usually a DNA binding protein, to stimulate the use of the promoter by RNA polymerase (25). In the simplest cases, class I activators bind upstream from the promoter and, like the UP element sequence, interact with the C-terminal domain of the RNA polymerase alpha subunit (18), whereas class II activators bind near the –35 region and most often interact with region 4 of the sigma subunit (16).

Class II activators stimulate different kinetic steps of promoter utilization. Promoter utilization by RNA polymerase can be divided into several discrete steps (31). The first is the

initial binding or recruitment of RNA polymerase to the promoter to form the so-called closed complex. Next, the closed complex undergoes isomerization to form the open complex in which the DNA strands are unwound. Subsequently, the initiating nucleotides are added to the transcript. The binding of class II activators to sigma region 4 can stimulate the initial binding of RNA polymerase or, as is the case for bacteriophage lambda cI activation of promoter P_{rm} , stimulate the transition from the closed to the open complex (30).

The *Bacillus subtilis* transcriptional activator Spo0A interacts with region 4 of σ^A , the primary sigma factor, to stimulate the activity of the *spoIIG* promoter (2, 9, 10, 28, 46). Spo0A, a classical two-component type response regulator in *B. subtilis*, is the master regulator of transcription at the onset of endospore formation (24). The activity of Spo0A is regulated by a complex phosphorelay system that culminates in the phosphorylation of Spo0A (11). Phosphorylated Spo0A (Spo0A~P) binds to a specific DNA sequence found in or near promoters to repress or activate these promoters (5, 32, 37, 38, 50, 51). The best-studied example of promoter activation by Spo0A~P is that of the *spoIIG* promoter, which directs the transcription of a two-gene operon that encodes a sporulation-essential secondary sigma factor, σ^E , and a protease required for σ^E activation (3–5, 10, 27, 28, 41, 42, 44–48). The *spoIIG* promoter is used by σ^A RNA polymerase, but promoter activity requires Spo0A~P, which binds at two pairs of Spo0A binding sites centered approximately 89 bp (site 1) and 43 bp (site 2) upstream from the start site of transcription (Fig. 1). Spo0A~P stimulates *spoIIG* promoter activity at a step subsequent to the initial binding of RNA polymerase to the promoter (47, 48). Indeed, RNA polymerase bound to the *spoIIG* promoter facilitates the binding of Spo0A~P to the promoter (48). Although Spo0A~P does not act to recruit RNA polymerase to the promoter, it also does not lower the activation energy of DNA melting (47). Therefore, Seredick and Spiegelman (48) previously proposed that Spo0A~P stimulates open complex formation by stabilizing a second closed complex that results

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[▽] Published ahead of print on 22 February 2008.

FIG. 1. The *spoIIG* promoter. Shown is the sequence of the *spoIIG* promoter (nontemplate strand). The start point of transcription is indicated as +1, with transcription proceeding left to right. The -10 DNA element recognized by σ^A is indicated in uppercase type, as is the -35 -like sequence centered at position -40 . The Spo0A binding sites (consensus sequence of $5'$ -TTGCA- $3'$) are indicated by arrows over the sequences. The lightning bolts above the sequence indicate the approximate positions of the major cleavage sites in the nontemplate strand observed in the FeBABE cleavage experiments, and those below the sequence indicate cleavage sites in the template strand. The cleavage sites indicated with open symbols indicate the absence of Spo0A, whereas the closed symbols indicate the presence of Spo0A.

after the initial binding of RNA polymerase and Spo0A~P at the promoter.

The *spoIIG* promoter has an unusual architecture in that a sequence similar to a consensus -35 element, normally contacted by sigma region 4, is separated from the -10 region element by 22 bp rather than the typical 17 or 18 bp (Fig. 1). Moreover, one of the sites (site 2) in the promoter to which Spo0A~P binds overlaps with this -35 -like sequence. Genetic evidence from our laboratory indicates that Spo0A~P interacts with region 4 of σ^A (2, 28). Furthermore, these genetic results define the putative interacting surfaces of Spo0A~P and σ^A . However, molecular modeling of the interacting surfaces of Spo0A~P and σ^A on DNA show that σ^A region 4 and Spo0A~P cannot bind simultaneously to the -35 -like element (28). These results led us to postulate that the binding of Spo0A~P to the -35 -like element of the *spoIIG* promoter repositions σ^A region 4 on the promoter to the region located 18 bp upstream from the -10 region (28). This repositioned RNA polymerase-promoter complex may be the stabilized closed complex intermediate posited previously by Spiegelman and colleagues to contribute to the Spo0A~P stimulation of *spoIIG* promoter activity. To test whether σ^A region 4 is repositioned on the promoter upon the binding of Spo0A~P, we used a DNA cleavage reagent to mark the location of σ^A region 4 on the DNA. We found that σ^A region 4 is positioned 4 bp downstream from the -35 -like element when Spo0A is bound to the *spoIIG* promoter-RNA polymerase complex.

MATERIALS AND METHODS

General microbiological methods. Routine microbiological manipulations and procedures were carried out by standard techniques as described previously (15). The concentrations of antibiotics used for selection were 5 μ g/ml chloramphenicol, 100 μ g/ml spectinomycin, 100 μ g/ml ampicillin, and 10 μ g/ml kanamycin. Competent cells were prepared and transformed by the two-step method as described previously (15).

Mutagenesis, plasmids, and assays of in vivo promoter activity. The Quick-Change site-directed mutagenesis kit (Stratagene, CA) was used to mutate *sigA* to produce an allele, *sigA*(G340C), encoding a single-amino-acid substitution of cysteine for glycine at position 340 (G340C) of σ^A . This allele of *sigA* was cloned into *B. subtilis* integration vector pJB2 (10) and in an *Escherichia coli* *sigA* expression vector, pCD2 (13). The oligonucleotide for the quick-change mutagenesis consisted of complementary 29 bp with the changed nucleotide centered in the middle of the primer. The resulting plasmids were checked for the presence of the appropriate mutations by sequencing. The *tms* promoter was generated from the *Bacillus subtilis* genome using oligonucleotides *tmsF* ($5'$ -A GCAAGGACTGCTGAAAGGGCTG- $3'$) and *tmsR* ($5'$ -GATTTTCATTCTCGT TCCTTGTCAGCCGC- $3'$) by PCR. The product was then cloned into plasmid pCR2.1 using the Topo TA cloning kit (Invitrogen, Carlsbad, CA).

The pCB2-derived plasmid carrying the G340C *sigA* allele was used to replace the wild-type *sigA* allele in *B. subtilis* JH642 and to isolate isogenic derivatives

containing *P_{tms}*- or *P_{spoIIG}*-*lacZ* transcriptional fusions as described previously (10). These strains were cultured in duplicate in Difco sporulation medium (DSM) containing 5 mg/ml chloramphenicol. Two 300- μ l aliquots of each culture were collected, one to measure the optical density and the other to assay β -galactosidase activity. Enzymatic activity was averaged from three independent experiments and calculated as described previously (23).

Protein preparation and in vitro footprinting assays. The C-terminal domain of Spo0A was prepared as described previously (14). RNA polymerase holoenzyme was prepared as described previously (1, 36). Core RNA polymerase was isolated from the holoenzyme preparation using a method described previously (1, 36). The G340C-substituted σ^A was purified after production in *E. coli* as described previously (13).

p-Bromoacetamidobenzyl-EDTA-Fe (FeBABE)-derivatized σ^A was prepared essentially as described previously (29). In brief, purified G340C-substituted σ^A was dialyzed in FeBABE conjugation buffer (10 mM MOPS [morpholinepropanesulfonic acid] [pH 8.0], 0.5 mM EDTA, 100 mM NaCl, 5% glycerol). FeBABE was purchased from Dojindo Laboratories. Conjugation was initiated by mixing 100 μ M sigma A (final concentration) in 700 μ l conjugation buffer with 1 mM FeBABE (final concentration). The samples were incubated for 1 h at room temperature, and the reaction was stopped by quenching the mixture with an equal volume of conjugation buffer. The efficiency of conjugation was determined by estimating free side chains of both conjugated and unconjugated proteins with the fluorescent reagent CPM [7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin] (Molecular Probes) as described previously (20). The conjugated FeBABE was then dialyzed into a solution containing 20 mM HEPES (pH 8.0), 50 mM potassium glutamate, 5 mM MgCl₂, 1 mM dithiothreitol, and 0.5 mg/ml bovine serum albumin. Samples were stored at -20°C after the addition of 50% glycerol.

DNA templates for footprinting were prepared by PCR as described previously (14) using the following oligonucleotide primers: *spoIIG*-For ($5'$ -AGTGG AAAAAAGCTGCCGTCATTGGCAGC- $3'$), *spoIIG*-REV ($5'$ -CTTTCCTCA GTCTACACTTTTAGAT- $3'$), *tms*-FOR ($5'$ -AGCAAGGACTGCTGAAAGG GCTG- $3'$), *tms*-REV ($5'$ -GATTTTCATTCTCGTTCTGTCCAGCCGC- $3'$), *spoIIG*-100FOR ($5'$ -TATATCCTCTCATTATACTTC), and *spoIIG*-15REV ($5'$ -CAAGCTCTGTGGGAAAGTCTG- $3'$). In each set of reactions, one oligonucleotide primer (50 pmol) was labeled with [γ -³²P]ATP by use of T4 polynucleotide kinase. The probe was separated from unincorporated nucleotides with a G-25 MicroSpin column (Amersham Pharmacia Biotech, Piscataway, NJ). The purified labeled probe was used in a PCR mixture containing 36 pmol of unlabeled primer and Herculase DNA polymerase (Stratagene). The PCR product was purified by elution from a G-50 MicroSpin column (Amersham Pharmacia Biotech, Piscataway, NJ).

To form holoenzyme to be used in the footprinting experiments, core RNA polymerase was incubated with a threefold molar excess of the FeBABE-derivatized σ^A on ice for 30 min. Reactions were initiated by incubating end-labeled DNA probes with 100 nM RNA polymerase in a 35- μ l reaction mixture containing 20 mM HEPES (pH 7.9), 50 mM potassium glutamate, and 0.1 mM EDTA for 15 min at 37°C . After the initial incubation with RNA polymerase, 100 nM Spo0A was added to the appropriate reaction mix, and the reaction mix was incubated for an additional 10 min. DNA cleavage was then initiated by the addition of a 5 mM concentration each ascorbate and hydrogen peroxide, and the reaction was allowed to proceed for 5 min at 37°C . The reaction was quenched with a buffer composed of 0.1 M thiourea, 1.0 M Tris (pH 8.0), 0.1 mg/ml calf thymus DNA, 0.2% sodium dodecyl sulfate, and 10 mM EDTA. Quenched samples were precipitated with 2 volumes of 95% ethanol, dissolved in formamide loading buffer (10 mM Tris [pH 8.0]-10 mM EDTA-97% form-

FIG. 2. Effect of a σ^A G340C substitution on transcription from the *tms* and *spoIIG* promoters. *B. subtilis* strains carrying a G340C substitution in *sigA* and *lacZ* transcriptional fusions to P_{tms} or P_{spoIIG} were cultured in liquid DSM. Samples were harvested at half-hourly intervals beginning 1 h before the end of the exponential growth phase, which is the onset of sporulation (time zero [T_0]), and assayed for β -galactosidase accumulation. Two independent cultures were assayed for each strain, and the averages are shown. (A) *spoIIG*-*lacZ* expression in *B. subtilis* strains EUAKB24 (wild-type σ^A) (closed circles) and EUAKB333 (G340C σ^A) (open circles). (B) *tms*-*lacZ* expression in *B. subtilis* strains EUAKB203 (wild-type σ^A) (closed circles) and EUAKB335 (G340C σ^A) (open circles).

amide, 0.1% sodium dodecyl sulfate, 0.1% xylene cyanol, 0.1% bromophenol blue), and subjected to electrophoresis in an 8% (wt/vol) polyacrylamide gel containing 8 M urea. The dried gel was then examined by PhosphorImager analysis.

RESULTS

FeBABA-tagged σ^A demonstrates close contact between region 4 and the -35 region of the *tms* promoter. We essentially used the same strategy to map the proximity of σ^A region 4 to promoter DNA as that described previously by Bown and his colleagues to map region 4 of *E. coli* σ^{70} on the *gal* operon promoter (6). They used the DNA cleavage agent FeBABA covalently tethered to a single cysteine (Cys581) in region 4 of a mutant form of σ^{70} . This cysteine-substituted σ^{70} was functional, and the RNA polymerase reconstituted with FeBABA-tagged Cys581 σ^{70} formed open promoter complexes in vitro (7). The activation of these complexes produced the cleavage of specific bases that were in close proximity to Cys581 of σ^{70} .

B. subtilis σ^A contains no cysteines; therefore, the preparation of a mutant form of σ^A containing a single cysteine required a substitution of cysteine for the glycine at position 340, the position in σ^A homologous to position 581 in σ^{70} . We anticipated that the mutant form of σ^A would be functional based on previous studies of σ^{70} . We examined the effects of the mutant *sigA* allele that encoded the G340C-substituted σ^A and found that the growth rate of the mutant strain was similar to that of the parental strain (data not shown). Moreover, we examined the effects of the G340C substitution on the expression of transcriptional fusions of *lacZ* to a Spo0A-independent promoter, P_{tms} , and to the Spo0A-dependent promoter, P_{spoIIG} in strains cultivated in sporulation medium (DSM). We found that the level and pattern of P_{tms} -*lacZ* expression were similar in the parental and G340C mutant *B. subtilis* strains (Fig. 2). Moreover, the expression levels of the Spo0A-dependent P_{spoIIG} -*lacZ* fusion were also similar in the parental and G340C mutant *B. subtilis* strains (Fig. 2).

Our next goal was to tag the mutant form of σ^A with FeBABA

and to determine whether RNA polymerase reconstituted with FeBABA-conjugated σ^A binds to and cleaves promoter DNA at positions known to be in close proximity to region 4. For our in vitro studies, we purified G340C-substituted σ^A from an *E. coli* expression strain. We conjugated FeBABA to the cysteine at position 340 of the purified G340C-substituted σ^A and showed that the FeBABA-conjugated σ^A , when reconstituted with core RNA polymerase, activated transcription from both the *tms* and *spoIIG* promoters in vitro (data not shown). To show that the RNA polymerase containing the FeBABA-conjugated σ^A bound and cleaved promoter DNA at positions known to be in close proximity to σ^A region 4, we examined the effects of FeBABA-directed cleavage of the *tms* promoter. The *tms* promoter (P_{tms}) from *B. subtilis* was chosen as a control because P_{tms} is used by σ^A RNA polymerase, and previous genetic suppression studies showed that arginine at position 347 (R347) in σ^A region 4 interacts with the base pair at position -34 of P_{tms} (26); therefore, the interaction of σ^A region 4 with this promoter appears to be identical to the interaction of σ^{70} region 4 with *E. coli* promoters. End-labeled DNA fragments containing the *tms* promoter were incubated with RNA polymerase containing the FeBABA-conjugated σ^A or in control reactions where the RNA polymerase was omitted. The activation of FeBABA resulted in the cleavage of the DNA as seen by autoradiography after electrophoresis of the fragments through polyacrylamide gels containing urea. The major cleavage products on the template strand resulted from cleavage at about position -38 relative to the start point of transcription, with minor products seen from cleavage near position -29 or -30 (Fig. 3). The major cleavage products on the nontemplate strand resulted from cleavages between positions -43 and -36 (Fig. 3). From these results, we concluded that the RNA polymerase-promoter complex region 4 of the FeBABA-tagged σ^A is positioned, as expected, in close proximity to the -35 region of the *tms* promoter.

Spo0A causes repositioning of σ^A region 4 on the *spoIIG* promoter. The results described above encouraged us to examine where σ^A region 4 is positioned in the RNA polymerase-pro-

FIG. 3. FeBABE footprinting of the *tms* promoter. Shown are DNA cleavage patterns at the *tms* promoter on the template (left) and the nontemplate (right) strands using σ^A -RNA polymerase (RNAP) tagged with FeBABE at position 340 in the presence and absence of Spo0A. The sequencing products generated using the same labeled primer are loaded in lanes a to d, and the letters above the lanes indicate the terminating dideoxynucleotides used in the reaction. Arrows point to cleavage products resulting from the hydroxyl radical-mediated cleavage of promoter DNA in the presence of FeBABE-derivatized σ^A RNA polymerase and Spo0A (lane e), FeBABE-derivatized σ^A RNA polymerase alone (lane f), or no protein (lane g). The numbers to the left of each panel indicate the positions relative to the start point of transcription. Shown below is the sequence of the *tms* promoter (nontemplate strand), with the start point of transcription indicated as +1 and other positions relative to the start point indicated above the sequence. The -35 element is shown in boldface type. The lightning bolts indicate the approximate locations of cleavages in nontemplate (above the sequence) or template (below the sequence) strands.

motor complex on the *spoIIIG* promoter and to examine the effect of Spo0A on its positioning. Again, end-labeled DNA fragments containing the *spoIIIG* promoter were incubated with RNA polymerase containing the FeBABE-conjugated σ^A or in control reactions where the RNA polymerase was omitted. The major cleavage products on the template strand resulted from cleavage at about position -45 relative to the start point of transcription, with minor products seen from cleavage about 10 bp downstream near position -35 (Fig. 4). The major cleavage products on the nontemplate strand resulted from cleavages centered near position -40 (Fig. 4). These cleavages are near the -35 -like element in the *spoIIIG* promoter (Fig. 1).

We also examined the effect of adding Spo0A to the polymerase promoter complexes. Here, we used the C-terminal domain of Spo0A, which has been shown to activate transcription independently of phosphorylation and has been used in previous studies as a surrogate for Spo0A~P (21). Remarkably, the addition of Spo0A to the complexes changed the

cleavage patterns. In the presence of Spo0A and RNA polymerase, the major products resulted from cleavage at about position -41 , and lesser cleavages near position -31 , on the template strand. The major cleavage products of the nontemplate strand resulted from cleavages near position -37 , with minor cleavages upstream and downstream. The addition of Spo0A to the RNA polymerase complex on the *tms* promoter had no effect on the positions of cleavage (Fig. 3), indicating that the effect of Spo0A was specific to the RNA polymerase-*spoIIIG* promoter complex. From these results, we conclude that σ^A region 4 in RNA polymerase bound to the *spoIIIG* promoter lies in close proximity to the -35 -like element centered at position -40 . However, in a complex of RNA polymerase and Spo0A on the *spoIIIG* promoter, σ^A region 4 lies in close proximity to base pairs located about 4 bp downstream from the -35 -like element. RNA polymerase and Spo0A bind and form a closed complex on a truncated version of the *spoIIIG* promoter in which the -10 and downstream regions

FIG. 4. FeBABE footprinting of the *spoIIIG* promoter. Shown are the DNA cleavage patterns at the *spoIIIG* promoter on the template (left) and the nontemplate (right) strands using σ^A -RNA polymerase (RNAP) tagged with FeBABE at position 340 in the presence and absence of Spo0A. The sequencing products generated using the same labeled primer are loaded in lanes d to g, and the letters above the lanes indicate the terminating dideoxynucleotides used in the reaction. Arrows point to cleavage products resulting from the hydroxyl radical-mediated cleavage of promoter DNA in the presence of no protein (lane a), FeBABE-derivatized σ^A RNA polymerase alone (lane b), or FeBABE-derivatized σ^A RNA polymerase and Spo0A (lane c). The numbers to the right of each panel indicate the positions relative to the start point of transcription.

are deleted but do not form open complexes (48). Therefore, we used FeBABE cleavage experiments to map the position of σ^A region 4 in complexes on a similar truncated promoter fragment missing its -10 element and downstream sequences. Again, end-labeled DNA fragments containing the truncated *spoIIIG* promoter were incubated with RNA polymerase containing FeBABE-conjugated σ^A and Spo0A or in control reactions where the Spo0A was omitted. The major cleavage products on the nontemplate strand of the RNA polymerase-promoter complex resulted from cleavage at positions near position -45 relative to the start point of transcription (Fig. 5). This cleavage pattern was very similar to that seen with the full-length promoter (Fig. 4). The addition of Spo0A to the RNA polymerase-truncated promoter complex resulted in groups of cleavage products near three positions, positions -48 , -41 , and -29 . This pattern of cleavages is reminiscent of the pattern seen with the full-length promoter complex (Fig. 4), except that the vast majority of cleavage products from the full-length promoter were the result of cleavages near position -41 , while the pattern of cleavages of the truncated promoter included a more or less equal distribution of cleavages at each of the three positions. We speculate that the subtle differences in cleavage patterns of RNA polymerase-Spo0A complex on the truncated promoter and on the full-length promoter reflect differences in the wrapping of the DNA through the complex.

FIG. 5. FeBABE footprinting of a truncated *spoIIIG* promoter. The figure shows the DNA cleavage patterns at the truncated (-10 and downstream regions deleted) *spoIIIG* promoter (nontemplate strand) using σ^A -RNA polymerase (RNAP) tagged with FeBABE at position 340 in the presence and absence of Spo0A. The dideoxycytosine sequencing products generated using the same labeled primer are loaded in lane a. Arrows point to cleavage products resulting from the hydroxyl radical-mediated cleavage of promoter DNA in the presence of no protein (lane b and e), FeBABE-derivatized σ^A RNA polymerase alone (lane c), or FeBABE-derivatized σ^A RNA polymerase and Spo0A (lane d). The numbers to the left indicate the positions relative to the start point of transcription.

Nevertheless, the similarities in the positions of cleavages in both complexes lead us to conclude that σ^A region 4 is positioned at similar regions in the truncated and full-length promoters and that Spo0A changes the position of σ^A region 4 similarly on both the full-length and truncated promoters.

DISCUSSION

Our previous genetic analyses indicated that Spo0A~P interacts with σ^A region 4, and molecular modeling of their interacting surfaces showed that σ^A region 4 was unlikely to simultaneously interact with Spo0A~P and bind to the -35 -like sequence in the promoter (28). Therefore, we hypothesized that σ^A region 4 would be displaced downstream from the -35 -like sequence by Spo0A~P. Here, we tested this hypothesis by specifically tagging σ^A region 4 with a DNA cleavage reagent and mapping its position on the DNA. We first tested whether *B. subtilis* RNA polymerase reconstituted with the FeBABE-derivatized σ^A could be used to map σ^A region 4 on an Spo0A-independent promoter. Previous genetic suppression studies provided strong evidence that σ^A region 4 interacts with the -35 region sequence of the *tms* promoter in a manner similar to that of σ^{70} region 4 of *E. coli* polymerase with its cognate promoters (26). In our FeBABE cleavage experiments, we found that the binding of the polymerase to the *tms* promoter resulted in cleavages immediately upstream and within the -35 element to produce patterns very similar to those seen by Bown and coworkers previously in experiments with the well-characterized FeBABE-derivatized *E. coli* RNA polymerase (7). The similarity of the cleavage locations on the *tms* promoter by our derivatized *B. subtilis* RNA polymerase and the cleavage patterns seen with the homologous *E. coli* polymerase provides strong support for our contention that FeBABE-derivatized *B. subtilis* RNA polymerase can be used to precisely map the location of σ^A region 4 on the DNA.

In reactions with the *spoIIG* promoter in which we omitted Spo0A, the cleavage patterns on the promoter were similar to that on the *tms* promoter in that the major cleavages were located near the upstream side of the -35 -like element. However, the consensus -35 element in the *spoIIG* promoter is centered about 40 bp upstream from the start point of transcription, whereas the -35 element of the *tms* promoter is located only 34 bp upstream from its start site of transcription. We conclude that σ^A region 4 is positioned near the -35 elements of *P_{tms}* and *P_{spoIIG}* in the absence of Spo0A. The addition of Spo0A to reaction mixtures containing the *spoIIG* promoter changed the cleavage sites, resulting in cleavages about 4 bp nearer the start point of transcription. Evidently, σ^A region 4 in these RNA polymerase-promoter complexes was displaced downstream by 4 bp. Two consequences of this displacement seem obvious. The first is that σ^A region 4 is no longer positioned over the consensus -35 element, where Spo0A would be bound, but, rather, is probably positioned to interact with a GAC sequence centered at position -35 . Although this sequence is partially similar to the consensus -35 element, σ^A region 4 probably does not interact strongly or specifically with this sequence, as Seredick and Spiegelman (48) showed previously that base substitutions in this sequence have little effect the stability of RNA polymerase binding. The second consequence is that the displacement of σ^A region 4 by

Spo0A results in the movement of σ^A region 2, probably to a position near the -10 element. This prediction follows because sigma is held relatively rigidly within RNA polymerase (i.e., in the holoenzyme, the spacing between sigma regions 2 and 4 is "roughly consistent with their binding sites on the DNA") (34). The new position of σ^A region 4, centered at about position -35 , is separated by 18 bp from the -10 element, nearly optimal spacing for promoter activity. Thus, the repositioning of RNA polymerase by Spo0A would be expected to facilitate open complex formation.

Seredick and Spiegelman (48) previously proposed that Spo0A stabilizes a second closed complex that precedes the formation of the open complex. They studied the binding of RNA polymerase and Spo0A to a truncated *spoIIG* promoter that was missing its -10 region and, therefore, could form only closed complexes. We compared the positioning of RNA polymerase on a truncated promoter to that on full-length promoter complexes. We found similar, but not identical, FeBABE cleavages with a truncated *spoIIG* promoter, indicating that the interaction with the -10 region is not essential for the Spo0A-stimulated repositioning of RNA polymerase on the promoter. Therefore, the repositioned RNA polymerase of the Spo0A RNA polymerase promoter complexes seen in our FeBABE cleavage experiments may be the proposed stabilized second closed complex.

Our results and those of Spiegelman and colleagues lead us to our current model of Spo0A stimulation of *spoIIG* promoter utilization, which involves several steps. RNA polymerase binds to the *spoIIG* promoter in the absence of Spo0A, probably by the interaction of the C-terminal domain of the alpha subunits to DNA upstream from the promoter and by the interaction of σ^A region 4 with the -35 -like sequence centered at position -40 . Although we have not shown that this first polymerase-promoter complex is an obligate intermediate, as opposed to a dead-end product, Seredick and Spiegelman previously showed that the bound RNA polymerase facilitates the binding of Spo0A to the promoter (48). Therefore, it seems unlikely to us that RNA polymerase completely releases from the promoter upon the binding of Spo0A. Spo0A binding displaces σ^A region 4 from the -35 -like sequence, displacing sigma and RNA polymerase to a position 4 bp downstream. During this movement on the promoter, the RNA polymerase is probably tethered to the upstream DNA by the C-terminal domain of the RNA polymerase alpha subunit (48). Stabilization of the RNA polymerase at this new position probably allows open complex formation by the interaction of a properly aligned σ^A region 2 with the -10 region sequences of the promoter.

The studies of the *E. coli gal* promoter reported previously by Bown et al. (6) served as a model for our approach to mapping the repositioning of RNA polymerase by a transcriptional activator; however, they found that cyclic AMP receptor protein did not cause a major change in the conformation or repositioning of RNA polymerase. Bown et al. (6) concluded that cyclic AMP receptor protein acted to recruit σ^{70} region 4 to the promoter -35 element. We suggest here that Spo0A stimulates the activity of a prebound RNA polymerase-*spoIIG* promoter complex, but the mechanism involves the recruitment of RNA polymerase to a new position on the promoter. Spo0A acts to stabilize the RNA polymerase at this new position on the promoter and thus acts similarly to the mechanism

proposed for cI activation of lambda phage promoter P_m (17, 25, 35, 39). In both cases, the specific interaction of an activator protein with the sigma factor stabilizes a transition intermediate to stimulate the rate of transcription initiation.

ACKNOWLEDGMENTS

We gratefully acknowledge Roy Doi for the *sigA* expression strain. This work was supported by Public Health Service grant GM54395 from the National Institute of General Medical Sciences.

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