Activation of the *Bacillus subtilis* spoIIG Promoter Requires Interaction of Spo0A and the Sigma Subunit of RNA Polymerase

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*B. subtilis* Spo0A activates transcription from both σ^H^- and σ^A^-dependent promoters. Baldus et al. (2) identified two amino acid substitutions in the carboxyl terminus of σ^A^, K356E and H359R, that specifically impaired Spo0A-activated transcription in vivo. To test the model in which the K356E and H359R substitutions in σ^A^- interfere with the interaction of Spo0A and σ^A^, we examined the effects of alanine substitutions at these positions in σ^A^- on σ^A^-'s ability to direct transcription in vivo and in vitro. We found that alanine substitutions at these positions specifically reduced expression from the σ^A^-dependent, Spo0A-dependent promoters, spoIIG and spoIIIE, in vivo. Furthermore, we found that stimulation of spoIIG promoter activity by Spo0A in vitro was reduced by the single substitutions H359A and H359R in σ^A^-.

A growing body of evidence supports the idea that site-specific DNA binding proteins activate promoters in bacteria by contacting RNA polymerase (reviewed in reference 4). The site on RNA polymerase that is contacted by the activator protein appears to depend in part on the site on DNA at which the activator protein is bound (16). The *Escherichia coli* cyclic AMP receptor protein (CAP) interacts with the C-terminal domain (CTD) of the RNA polymerase alpha subunit when CAP is bound at a site centered about 61 bp upstream from the transcription start point (−61) (reviewed in reference 11), whereas CAP bound at a site centered at position −41 activates transcription by contacting the N-terminal domain of the α subunit (25; reviewed in reference 5). Other activator proteins contact the σ subunit of RNA polymerase, FNR (a protein that is structurally similar to CAP), PhoB, MalT, and cI from phage lambda bind to sites that overlap the −35 region of promoters, and probably contact the σ subunit of RNA polymerase (reference 5 and references therein; 9, 22–24). Some promoters contain multiple binding sites for activators. In some of these cases the two coactivators appear to interact directly with the RNA polymerase (6, 18, 29). At other promoters one coactivator affects DNA binding by the second coactivator, which is the only one to make direct contact with RNA polymerase (26).

Spo0A from *Bacillus subtilis*, which is required for the initiation of sporulation, binds the spoIIG promoter at two sites known as A boxes, centered at positions −37 and −87 (reviewed in reference 15; 1, 27). Binding of Spo0A at these sites stimulates utilization of the spoIIG promoter by RNA polymerase containing σ^A^, a homolog of *E. coli* σ^70^- (1, 3, 20, 27, 28). It is not known whether spoIIG promoter activation involves interactions between Spo0A and RNA polymerase. Baldus et al. (2) found that spoIIG promoter activity was reduced in mutants of *B. subtilis* in which σ^A^- contained one of two single amino acid substitutions (glutamate substituted for lysine at position 356, K356E, or arginine substituted for histidine at position 359, H359R). These observations led to the suggestion that spoIIG promoter activation involves an interaction between Spo0A and σ^A^-.

The two amino acid substitutions in σ^A^- that reduced spoIIG promoter activity lie near the region of σ^A^- that makes sequence-specific contacts with the base pairs at the −35 region of its cognate promoters (Fig. 1). Other amino acid substitutions in this region of σ^A^- and other sigma factors have been shown to change the specificity of promoter utilization by RNA polymerase containing these mutant sigma factors (12, 19, 32). An alternative explanation for the results of Baldus et al. (2) could be that K356E or H359R slightly changes the specificity of promoter recognition by RNA polymerase, resulting in changes in gene expression that block the initiation of sporulation, and indirectly reduces spoIIG promoter activity. To determine whether the amino acyl residues at positions 356 and 359 of σ^A^- are required specifically for activation of the spoIIG promoter by Spo0A, we examined the effects of single mutations to alanine at these positions on promoter utilization in vivo. We also examined the effects of amino acid substitutions at these positions on stimulation of spoIIG promoter activity in vitro by purified Spo0A.

Mutant sigA alleles were made in vitro by using a multistep PCR procedure described by Cormack (8) and used to replace the wild-type (wt) allele in the *B. subtilis* chromosome by transformation of strain JH642 to kanamycin resistance as described previously (2). Strains EUC9603 and EUC9604 express the H359A and K356A forms of σ^A^, respectively. The isogenic wt parent of these strains produced 4 × 10^8 heat-resistant spores/ml, as did EUC9604. Sporulation of EUC9603 was severely reduced, resulting in only 1.3 × 10^7 heat-resistant spores/ml.

To monitor the effects of each amino acid substitution on the activation of specific promoters, strain EUC9603, strain EUC9604, and the wt parent were lysogenized with SPβ specialized transducing phages that carried lacZ transcription fusions to one of four different promoters. We examined the accumulation of β-galactosidase in cultures of these strains during growth and sporulation as described previously (2) (Fig. 2). The H359A mutation completely abolished activity from the two Spo0A-dependent, σ^A^-dependent promoters, spoIIG (Fig. 2) and spoIIIE (data not shown). The K356A mutation...
Spo0A-dependent promoter

Spo0A-dependent promoter

spoIIG
tms
cubating the Spo0A-independent

Spo0A-independent

the unphosphorylated form of Spo0A (data not shown). Nonetheless, since the expression of Spo0A, since the amplification of the Spo0A-independent, tms promoter was unaffected by the H359A and K356A substitutions in α^70 (Fig. 2). There was also no effect of the amino acid substitutions in α^70 on the Spo0A-dependent, σ^70-dependent promoter tms was unaffected by the H359A and K356A substitutions in α^70 (Fig. 2). The results show that the side chains of H359 and K356 are important for wt levels of Spo0A-dependent spoIIG and spoIIE promoter activity. Additionally, these side chains are not required for activity of the α^70-specific, Spo0A-independent tms promoter. Moreover, it seems unlikely that these changes in α^70 affected expression of Spo0A, since the α^70-specific spoIIA promoter, whose activity requires Spo0A (1, 33), is active in these strains (Fig. 2).

To eliminate the possibility that the effects of these changes in α^70 on utilization of the spoIIG promoter were caused by some unanticipated indirect effect on α^70 activity, other than its ability to interact with Spo0A, we tested the effects of these mutations by in vitro transcription. We isolated the RNA polymerase α^70 holoenzyme (31) from EUC9605 (wt α^70), EUB9403 (H359R α^70) (2), and EUC9603 (H359A α^70) cell, harvested at late exponential phase in Luria-Bertani medium. The Spo0A activator protein required in our minimal in vitro system was purified from the overproducing E. coli strain JM109(pGO16) (1). However, Spo0A produced in E. coli is the unphosphorylated form, and its efficiency in activation of spoIIG transcription is very low (1, 3). Studies of the domain structure of Spo0A have revealed an amino-terminal domain which undergoes specific aspartate phosphorylation and a CTD able to specifically bind to DNA (13). We used the CTD Spo0A as the activator in the in vitro experiments. Preparation of this activation domain was performed by trypsin cleavage of Spo0A and subsequent separation of proteolytic products by affinity chromatography on heparin, as described in reference 13. For the same concentration, the CTD Spo0A was observed to stimulate three times more specific spoIIG transcription than the unphosphorylated form of Spo0A (data not shown).

In vitro transcription experiments were performed by co-incubating the Spo0A-independent tms promoter (10) and the Spo0A-dependent promoter spoIIG −38C (27) with wt α^70 holoenzyme or H359A α^70 holoenzyme in the presence of various concentrations (0 to 400 nM) of the CTD Spo0A (Fig. 3a). Regardless of CTD Spo0A concentrations, wt and H359A holoenzymes were able to generate tms transcripts with equal efficiencies (Fig. 3a). However, this was not the case for spoIIG transcription. To be able to more easily compare results from different enzyme preparations and from different incubations, we compared the amount of spoIIG transcript to the tms transcripts and plotted this ratio as a function of CTD Spo0A concentration.
concentrations (Fig. 3b). In reaction mixtures containing wt σ^- the spoIIG signal increased proportionally to the concentration of Sp0A until the highest concentration used in our experiment (400 nM). In contrast, spoIIG transcription was not stimulated as efficiently by CTD Sp0A in the reaction mixtures containing the H359A σ^- holoenzyme (Fig. 3). spoIIG transcription by the H359R σ^- holoenzyme was also not stimulated efficiently by CTD Sp0A (Fig. 3b). We observed a small decrease in the basal level of spoIIG transcription (without added CTD Sp0A) by both substitution-containing σ^- holoenzymes compared, but this effect (threefold with H359A and twofold with H359R) was not as large as the effects that these substitutions had on Sp0A-stimulated transcription.

Our results support the model in which spoIIG promoter activation requires an interaction between Sp0A and σ^-A. It is unlikely that the effects on promoter utilization of substitutions at positions 356 and 359 result from subtle changes in the promoter recognition specificity by σ^-A since substitutions of amino acids with large side chains (K356E and H359R) and smaller side chains (K356A and H359A) had similar effects on promoter utilization in vivo. Moreover, the effects on promoter utilization in vivo were not caused indirectly by some unknown effects on the cell’s physiology, since similar effects were seen when purified components were used to reconstruct transcription in vitro. Indeed, our observation that RNA polymerase containing the H359A form of σ^-A was not stimulated as efficiently as wt σ^-A RNA polymerase to use the spoIIG promoter in vitro strongly supports the model in which close contact between Sp0A and σ^-A is required for spoIIG promoter activation. It is unlikely that this alanine substitution could have distorted the structure of another subunit of the RNA polymerase or of the promoter DNA to which the polymerase was bound, since alanine contains no large side chain and since the H359A form of σ^-A efficiently directed transcription from the tms promoter. Therefore, since the effect of the H359A substitution was observed in a reaction mixture containing purified RNA polymerase and Sp0A, it is likely that the H359A substitution eliminates an interaction between σ^-A and Sp0A that is essential for efficient utilization of the spoIIG promoter. Moreover, it is also likely that Sp0A directly contacts σ^-A near position 359 because the alanine substitution is unlikely to have long-range effects on σ^-A structure.

The K356A substitution also reduced spoIIG promoter activity in vivo (albeit to a lesser degree than the effect of the H359A substitution). The strain containing the K356A mutation retained 50% of wt spoIIE activity and 17% of normal spoIIG activity. Though low, these amounts of promoter activation still produced a sporulation-proficient strain. When the lysine at 356 was mutated to a glutamic acid, a more dramatic Sp0A-specific effect was observed (2). These results are consistent with a model in which Sp0A contacts σ^-A at the region near positions 359 to 356. Both of these amino acids, K356 and H359, are located in a region of σ^-A (351 to 360) (Fig. 1) which presents a net positive charge. This region might electrostatically interact with a negatively charged subregion of Sp0A. A homologous region in σ^-S is suspected to be involved in electrostatic interactions with transcriptional activators. For example, transcriptional activation by CAP’ AR3 (a CAP derivative with a substitution of K52) at class II CAP-dependent promoters and by εf at the P_FK promotor may involve ionic interactions (reference 5 and references therein; 7, 22).

Our results indicate that an interaction between Sp0A and σ^-A is required for spoIIG promoter activation. Sp0A binds to two sites on the spoIIG promoter, and it is not known at which site Sp0A is bound when it contacts the 356-to-359 region of σ^-A. Other activators that bind at sites upstream from position...
-45 are thought to contact the α subunit of RNA polymerase, whereas those that bind near position -35 interact with either the α subunit or the α subunit (16; reviewed in references 4 and 5). Furthermore, since Spo0A binds to two sites on the spoIIG promoter, it is possible that Spo0A may make more than one contact with RNA polymerase when bound to the spoIIG promoter. We also note that the architecture of Spo0A-dependent promoters appears highly variable. Some of these promoters are used by RNA polymerase containing σS, others are used by σ70 holoenzymone, and they exhibit different organizations of the 0A boxes (reviewed in reference 30). This variety of architectures may indicate that multiple different interactions between Spo0A and RNA polymerase are possible and that Spo0A interacts with different subunits of the RNA polymerase. Multiple interactions of activators with RNA polymerase resulting in synergistic activation of promoter activity have been reported for E. coli promoters (6, 18, 29) but not for promoters in B. subtilis.

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REFERENCES


