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α-Helix E of Spo0A Is Required for σA- but Not for σH-Dependent Promoter Activation in Bacillus subtilis

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At the onset of endospore formation in Bacillus subtilis, the DNA binding protein Spo0A activates transcription from two types of promoters. The first type includes the spoIG and spoIIE promoters, which are used by σA-RNA polymerase, whereas the second type includes the spoIIA promoter, which is used by RNA polymerase containing the secondary sigma factor σH. Previous genetic analyses have identified specific amino acids in α-helix E of Spo0A that are important for activation of Spo0A-dependent, σA-dependent promoters. However, these amino acids are not required for activation of the σH-dependent spoIIA promoter. We now report the effects of additional single-amino-acid substitutions and the effects of deletions in α-helix E. The effects of alanine substitutions revealed one new position (239) in Spo0A that appears to be specifically required for activation of the σH-dependent promoters. Based on the results of a deletion mutation, we suggest that α-helix E in Spo0A is not directly involved in interaction with σH-RNA polymerase.

Initiation of endospore formation in Bacillus subtilis is controlled by the DNA binding protein Spo0A, which activates transcription from several promoters, including spoIG (11), spoIIE (17), and spoIIA (2, 15), by binding to sites near the ~35 region of these promoters. The spoIG and spoIIE promoters are used by σA-RNA polymerase, whereas the spoIIA promoter is used by RNA polymerase containing the secondary sigma factor σH. Previous studies conducted by Buckner et al. (1) and Hatt and Youngman (4) identified a 14-amino-acid region in the C terminus of Spo0A (from residues 227 to 240) important for activation of Spo0A-dependent, σA-dependent promoters. Specifically single-amino-acid substitutions at positions G227, I229, S233, F236, and V240 result in reduced ability to stimulate transcription of σA-specific promoters while having no effect on stimulation of σH-dependent promoters (1, 4). In addition to these mutations that specifically impair σA-dependent promoter activation, Buckner et al. (1) also report that a mutant form of Spo0A (S231F) suppresses the sporation defect of H359R and several other substitutions in α-helix E, a flexible helix in the C terminus of the protein that is positioned away from the core structure of the protein (7, 18) (Fig. 1). Taken together these results suggest that α-helix E perhaps contacts σA-RNA polymerase when bound to promoters to stimulate transcription. It is not known whether all the amino acid residues in α-helix E that are involved in stimulation of σA-directed transcription have been identified or whether any of the amino acids in α-helix E play a direct role in activation of σH-RNA polymerase. Therefore, we examined the effects of additional single-amino-acid substitutions and the effects of deletions in α-helix E on activation of σA- and σH-dependent promoters.

MATERIALS AND METHODS

Bacterial strains and culture media. Routine microbiological manipulations and procedures were carried out by standard techniques as described by Harwood and Cutting (3). The concentrations of antibiotics used for selection on Luria broth or Difco sporulation media (DSM) were 5 µg/ml for chloramphenicol, 100 µg/ml for spectinomycin, and 100 µg/ml for ampicillin. Cultures were grown in Luria broth, and sporulation was induced by nutrient exhaustion in DSM. Competent cells were prepared and transformed by the two-step method as described by Harwood and Cutting (3).

The B. subtilis strains used (Table 1) are all derivatives of JH642 and contain the trpC2 and phe-1 alleles. Plasmids derived from pCB2 (1) were used for inserting various mutations at the wild-type spo0A locus.

In order to create the spo0A deletion strain (EUAKB78), the 5'-flanking DNA of spo0A was PCR amplified with primers 0AUUSFOR (HindIII end) and 0ADSSREV (BstI end) and was cloned into HindIII-BstI-digested pCB3 (1) to generate plasmid pAK53. pAK53 was linearized with ScaI and was transformed into JH642 as previously described by Buckner et al. (1). Chromosomal DNA was isolated from a spectinomycin-resistant transformant by using the Quamp DNA Mini Kit (Qiagen Inc., Valencia, Calif.) and was subjected to PCR to determine if the gene replacement of spectinomycin for spo0A occurred. The following PCR primers were used in combination, 0AUUS5FOR and SpecREV or 0ADS2REV and SpecFOR, to confirm the allelic replacement of the spo0A locus.

The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, Calif.) was used to create mutations in spo0A that resulted in single alanine substitutions. Briefly, pCB2 was used to create single alanine substitutions from amino acid positions 234 to 239 of Spo0A. The combination of FOR and REV primers listed in Table 1 was used to make the single-amino-acid substitutions, and the resulting plasmids for each mutation were subjected to sequencing by using the 0AUUSFOR and 0ASCREV primers to ensure the presence of the desired mutation.

Overlapping PCR was used to create three different deletions within the coding sequence of Spo0A. In deletion 1, amino acids from positions 225 to 244 were deleted, and in deletion 2, amino acids from positions 233 to 241 were deleted, while in the third class of deletion amino acids from positions 229 to 241 were deleted. In the first round of PCR, the 5’ end of the coding sequence (from the starting methionine to either amino acid position S225, S233, or I229) was amplified with forward primer 0AUUSFOR in combinations with reverse primer 0ADSSREV, 0ADS4REV, or 0ADS3REV. This generated three PCR products that contained overlapping complementary regions at their 3’ end to primers.
FIG. 1. Ribbon diagram of the C-terminal, DNA binding domain of Spo0A. The structure of the Bacillus stearothermophilus Spo0A is based on coordinates from Lewis et al. (7) and is generated by using RasMol version 2.7.1.1. The α-helix E is colored blue, and the rest of the structure is red. Regions of the helix deleted in B. subtilis Spo0A are delineated by the numbered amino acid positions. Amino acid positions R226 and T243 correspond to amino acid positions R218 and S241 to S267 in B. stearothermophilus Spo0A. The structure of the Bacillus stearothermophilus Spo0A is based on coordinates from Lewis et al. (7) and is generated by using RasMol version 2.7.1.1. The α-helix E is colored blue, and the rest of the structure is red. Regions of the helix deleted in B. subtilis Spo0A are delineated by the numbered amino acid positions. Amino acid positions R226 and T243 correspond to amino acid positions R218 and S241 to S267 in B. stearothermophilus Spo0A. The structure of the Bacillus stearothermophilus Spo0A is based on coordinates from Lewis et al. (7) and is generated by using RasMol version 2.7.1.1. The α-helix E is colored blue, and the rest of the structure is red. Regions of the helix deleted in B. subtilis Spo0A are delineated by the numbered amino acid positions. Amino acid positions R226 and T243 correspond to amino acid positions R218 and S241 to S267 in B. stearothermophilus Spo0A. The structure of the Bacillus stearothermophilus Spo0A is based on coordinates from Lewis et al. (7) and is generated by using RasMol version 2.7.1.1. The α-helix E is colored blue, and the rest of the structure is red. Regions of the helix deleted in B. subtilis Spo0A are delineated by the numbered amino acid positions. Amino acid positions R226 and T243 correspond to amino acid positions R218 and S241 to S267 in B. stearothermophilus Spo0A. The structure of the Bacillus stearothermophilus Spo0A is based on coordinates from Lewis et al. (7) and is generated by using RasMol version 2.7.1.1. The α-helix E is colored blue, and the rest of the structure is red. Regions of the helix deleted in B. subtilis Spo0A are delineated by the numbered amino acid positions. Amino acid positions R226 and T243 correspond to amino acid positions R218 and S241 to S267 in B. stearothermophilus Spo0A. The structure of the Bacillus stearothermophilus Spo0A is based on coordinates from Lewis et al. (7) and is generated by using RasMol version 2.7.1.1. The α-helix E is colored blue, and the rest of the structure is red. Regions of the helix deleted in B. subtilis Spo0A are delineated by the numbered amino acid positions. Amino acid positions R226 and T243 correspond to amino acid positions R218 and S241 to S267 in B. stearothermophilus Spo0A. The structure of the Bacillus stearothermophilus Spo0A is based on coordinates from Lewis et al. (7) and is generated by using RasMol version 2.7.1.1. The α-helix E is colored blue, and the rest of the structure is red. Regions of the helix deleted in B. subtilis Spo0A are delineated by the numbered amino acid positions. Amino acid positions R226 and T243 correspond to amino acid positions R218 and S241 to S267 in B. stearothermophilus Spo0A. The structure of the Bacillus stearothermophilus Spo0A is based on coordinates from Lewis et al. (7) and is generated by using RasMol version 2.7.1.1. The α-helix E is colored blue, and the rest of the structure is red. Regions of the helix deleted in B. subtilis Spo0A are delineated by the numbered amino acid positions. Amino acid positions R226 and T243 correspond to amino acid positions R218 and S241 to S267 in B. stearothermophilus Spo0A. The structure of the Bacillus stearothermophilus Spo0A is based on coordinates from Lewis et al. (7) and is generated by using RasMol version 2.7.1.1. The α-helix E is colored blue, and the rest of the structure is red. Regions of the helix deleted in B. subtilis Spo0A are delineated by the numbered amino acid positions. Amino acid positions R226 and T243 correspond to amino acid positions R218 and S241 to S267 in B. stearothermophilus Spo0A. The structure of the Bacillus stearothermophilus Spo0A is based on coordinates from Lewis et al. (7) and is generated by using RasMol version 2.7.1.1. The α-helix E is colored blue, and the rest of the structure is red. Regions of the helix deleted in B. subtilis Spo0A are delineated by the numbered amino acid positions. Amino acid positions R226 and T243 correspond to amino acid positions R218 and S241 to S267 in B. stearothermophilus Spo0A.
produced expression of substitution of mutations F236A and T239A resulted in re-
similar to that of the wild-type strain (Table 3). However, 
These mutants also formed heat-resistant spores at frequencies 
spoIIG-lacZ had little effect on the expression of 
lactosidase was monitored during endospore formation. Three 
/H9252 activating spoIIA-lacZ had little to no effect on expression of 
spo0A functions as least as well as wild-type Spo0A in 
stimulation of spo0A-directed transcription and in repressing 
spoIIA-lacZ promoter, (an Spo0A-repressed promoter). We 
transduced these mutants with specialized SPB phage lysates that carried either fusions of 
spoIIG-lacZ (an Spo0A-activated, σ^A-dependent promoter), spoIIA-lacZ (an Spo0A-activated, σ^{II}-dependent promoter), or 
abrB-lacZ (an Spo0A-repressed promoter). We also isolated isogenic transductants of a strain containing a 
spectinomycin marker linked to the wild-type spo0A allele and of a strain carrying a deletion of the 
spo0A locus. All of the strains were cultured in DSM, and the accumulation of β-galactosidase was monitored during endospore formation. Three of the single alanine substitutions (L235A, G237A, and Y238A) had little effect on the expression of 
spoIIG-lacZ (Table 3). These mutants also formed heat-resistant spores at frequencies similar to that of the wild-type strain (Table 3). However, substitution of mutations F236A and T239A resulted in reduced expression of 
spoIIA-lacZ and spore formation (Table 3; Fig. 2). The T239A substitution caused increased expression of 
spoIIA-lacZ and had little to no effect on expression of 
abrB-lacZ (Table 3; Fig. 2). These latter results indicate that T239A replaced Spo0A functions as least as well as wild-type Spo0A in activating spoIIA transcription and in repressing 
abrB tran-

### RESULTS AND DISCUSSION

Identification of a new position in α-helix E of Spo0A that is required for σ^A-dependent promoter activation. To determine whether additional amino acids in α-helix E are involved in stimulation of σ^A-dependent promoters and if any of these amino acids play a role in stimulation of σ^{II}-directed transcription, we isolated mutants that produced single alanine substitutions at each position from 234 to 239 in Spo0A. To assay the effects of the single alanine substitutions in Spo0A on expression of Spo0A-regulated promoters, we transduced these mutants with specialized SPB phage lysates that carried either fusions of spoIIG-lacZ (an Spo0A-activated, σ^A-dependent promoter), spoIIA-lacZ (an Spo0A-activated, σ^{II}-dependent promoter), or 
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abrB tran-

### TABLE 3. Effects of alanine substitutions for amino acid residues 234 to 239 within wild-type *B. subtilis* Spo0A

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>No. of CFU (ml⁻¹)</th>
<th>No. of heat-resistant spores (ml⁻¹)</th>
<th>β-Galactosidase activity (Miller units)³ for:</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUAKB18</td>
<td>Isogenic wild type</td>
<td>9 × 10⁸</td>
<td>6 × 10⁹</td>
<td>IIGlacz</td>
</tr>
<tr>
<td>EUAKB78</td>
<td>Null</td>
<td>15 × 10⁸</td>
<td>No spores</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>EUAKB56</td>
<td>S234A</td>
<td>8 × 10⁸</td>
<td>11 × 10⁸</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>EUAKB55</td>
<td>L235A</td>
<td>5 × 10⁸</td>
<td>4 × 10⁸</td>
<td>265 ± 31</td>
</tr>
<tr>
<td>EUAKB57</td>
<td>F236A</td>
<td>9 × 10⁸</td>
<td>10 × 10⁸</td>
<td>31 ± 9</td>
</tr>
<tr>
<td>EUAKB54</td>
<td>G237A</td>
<td>15 × 10⁸</td>
<td>7 × 10⁸</td>
<td>39 ± 8</td>
</tr>
<tr>
<td>EUAKB20</td>
<td>Y238A</td>
<td>6 × 10⁸</td>
<td>3 × 10⁸</td>
<td>37 ± 10</td>
</tr>
<tr>
<td>EUAKB58</td>
<td>T239A</td>
<td>5 × 10⁸</td>
<td>15 × 10⁸</td>
<td>5 ± 1</td>
</tr>
</tbody>
</table>

³ Cultures were grown for 30 h at 37°C, serially diluted, and plated for counting.
⁴ Same cultures were heated for 20 min at 80°C, serially diluted, and plated for counting.
⁵ β-Galactosidase activity of the promoter fusions at 3 h after the end of the stationary phase. Shown is the average from two experiments.

![FIG. 2. Effect of substitution T239A on the expression of spoIIA-lacZ, spoIIG-lacZ, and abrB-lacZ transcriptional fusions. DSM cultures of each transduced strain, i.e., EUAKB18 (wild-type–0A), EUAKB78 (Null–0A), EUAKB58 (T239A–0A), and EUAKB78 (Null–0A), were harvested at hourly intervals beginning at about 1 h before the end of the exponential growth, which is indicated as 0 on the time scale. The collected samples were assayed for β-galactosidase activity indicated in Miller units.](https://example.com/fig2.png)
The effects of the deletions on expression of the Spo0A-regulated promoter-lacZ fusions. All three deletions abolished transcription from the spoIIA and spoIIG promoters (data not shown). However, the three deletion derivatives of Spo0A repressed expression of abrB-lacZ (data not shown), suggesting that these deletion derivatives of Spo0A retained their ability to bind DNA.

We expected that deletion of α-helix E would prevent activation of α^H-dependent promoters, since several amino acids in this region have been shown to be essential for α^H-dependent promoter activation. However, deletion of α-helix E also impaired the ability of Spo0A to activate α^H-directed transcription. In order to explore further the possibility that α-helix E is essential for stimulating α^H-directed transcription, we sought to identify intragenic suppressors of the deletions. A selection for sporulation-proficient derivatives of the α-helix E deletion strains failed, probably because sporulation would require restoration of both α^H-directed and α^H-dependent transcription. However, during this procedure we discovered that a single valine (GTT)-to-alanine (GCT) substitution at position 8 (V8A) in the deletion 1 derivative of Spo0A suppressed the effect of the deletion on α^H-dependent spoIIA-lacZ expression (Fig. 4). The V8A substitution did not suppress the effect of the deletion on spoIIG-lacZ (Fig. 4) expression, nor did it restore formation of heat-resistant spores (data not shown). Therefore, α-helix E in the V8A-substituted Spo0A is not required for activation of the α^H-dependent spoIIA promoter, while α-helix E is required for activation of the α^H-dependent spoIIG promoter.

Since α-helix E is not required for stimulation of α^H-directed transcription, at least not by the V8A-substituted Spo0A, how does the deletion of α-helix E affect α^H-directed transcription and how does the V8A substitution suppress the effect of the deletion on α^H-directed transcription? Immunoblot analyses of Spo0A accumulation during sporulation revealed that the deletion derivatives of Spo0A accumulated to levels that were at least two- to fourfold lower than that of wild-type Spo0A (data not shown). These lower levels of accumulation may have been caused by small decreases in stability of the deletion derivatives of Spo0A and may have been compounded by the requirement of Spo0A for stimulating transcription of its own structural
gene (14). However, the small decrease in accumulation of the Spo0A deletion derivatives probably played no role in the reduction of Spo0A-dependent promoter activation. Immunoblot analyses showed that the V8A substitution did not substantially increase the accumulation of Spo0A (data not shown). Nevertheless, the V8A substitution restored activation of the spoIIA promoter by the deletion 1 derivative Spo0A. One possible explanation for these results is that the V8A substitution increases the fraction of the Spo0A that is phosphorylated, which allows the protein to stimulate transcription more efficiently. Consistent with this hypothesis is the observation by Stephenson et al. (13) that N12 of Spo0A is critical for interaction with the Spo0E phosphatase. Two highly conserved aspartate residues, D10 and D11, that form part of the acid pocket at the phosphorylation site are located between N12 and V8 (6). Therefore, it is possible that the V8A substitution may reduce interaction with the Spo0E phosphatase, resulting in higher levels of Spo0A phosphorylation. The equivalent residue of Spo0A V8 is normally a hydrophobic residue in response regulator receiver domains and lies in the first element of the secondary structure, a β-strand that contributes to the positioning of the β1-α1 loop containing the residue D10-N12, and so this is our preferred explanation.

Other possible mechanisms by which the V8A substitution restores activation of σH-directed transcription by the α-helix E deletion derivative of Spo0A would include creation of an interaction between the N-terminal domain of Spo0A and RNA polymerase that compensates for an interaction with RNA polymerase that was lost upon deletion of α-helix E or a model in which the V8A affects interaction between the N- and C-terminal domains of Spo0A. We cannot eliminate the former model, but it seems unlikely that substitution of valine for alanine, which effectively removes side chain volume, would establish a new interaction between proteins and seems likelier that the alanine substitution would eliminate an interaction, such as between Spo0A and Spo0E. We also cannot completely eliminate the latter model. However, if the V8A substitution affects the interaction between the C- and N-terminal domains of Spo0A, the effect on the conformation of the C-terminal domain would likely be very small. This effect would not likely be great enough to compensate for the absence of α-helix E if this helix plays a direct role in stimulating σH-RNA polymerase. Therefore, we conclude that α-helix E in Spo0A probably is not directly involved in interaction with σH-RNA polymerase. If α-helix E does not interact with σH-RNA polymerase, then another region of Spo0A probably interacts with σH-RNA polymerase. Presently the best candidate for a region of Spo0A that interacts with σH-RNA polymerase is at the extreme C terminus, where amino acid substitutions at positions 257, 258, and 260 have been shown by Perego et al. (8) to reduce activation of the σH-dependent promoter spoIIA. However, as Rowe-Magnus et al. (9) discuss in their paper, they could not eliminate an indirect role for this region in activation of σH-dependent promoters.

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