Identification of a Second Region of the Spo0A Response Regulator of Bacillus subtilis Required for Transcription Activation

DEAN A. ROWE-MAGNUS,† MARTIN J. RICHER, and GEORGE B. SPIEGELMAN

Departments of Microbiology and Immunology and Medical Genetics, University of British Columbia, Vancouver, Canada V6T 1Z3

Received 13 September 1999/Accepted 11 May 2000

The Bacillus subtilis response regulator Spo0A stimulates transcription from a variety of stationary-phase and sporulation-specific promoters (7, 15, 23). Stimulation by Spo0A is mediated by the C-terminal domain, whose activity is blocked until the N terminus has been phosphorylated (1, 11, 15, 23). Spo0A is unusual in that it activates transcription from promoters transcribed by RNA polymerase holoenzyme containing either the σ^A or σ^H sigma factor (reviewed in reference 23).

One region of Spo0A that is required for transcription activation is between amino acids 227 and 240 in the C-terminal domain. Mutations in this region block stimulation of σ^A-dependent promoters, and this region has been proposed as a site of contact with the σ^A subunit (2, 4, 5, 12, 22). Spo0A-σ^A contact is supported by identification of mutations in both σ^A and σ^H that prevent transcription from Spo0A-dependent promoters but have no effect on transcription from Spo0A-independent promoters (2, 4, 22). Mutations in the σ^A contact region do not affect transcription from σ^H-dependent promoters, suggesting that Spo0A may have a separate contact region for σ^H (4, 12) or that it may activate transcription via different mechanisms at σ^A- and σ^H-dependent promoters.

Deletion of the C-terminal 15 residues of Spo0A generates a mutant blocked at stage 0 of sporulation (8). Substitution of either valine or glutamic acid for the alanine at position 257, which is the 11th amino acid from the C terminus, causes a sporulation-deficient phenotype and abolishes transcription from a variety of stationary-phase and sporulation-dependent promoters (2, 4, 22). Mutations in the C-terminal 15 residues of Spo0A (obtained from J. A. Hoch, Scripps Institute, San Diego, Calif.) was amplified and cloned into plasmid pDH32. Transformants resulting from Campbell-type recombination between the plasmid-borne spo0A gene and the chromosomal allele were selected, and 10 representatives from each transformation were examined for their ability to sporulate (6). We reasoned that if A257 was the only critical amino acid within the last 15 amino acids of the sequence, deletion of the 10 amino acids C terminal to A257 would not affect sporulation. As shown in Table 2, this was not the case for the DR2004 mutant, so we extended the analysis by carrying out valine-scanning mutagenesis of the 10C-terminal amino acids. Of the valine substitution mutants, DR2006 and DR2008, which carry the spo0A9V and spo0AL260V alleles, respectively, had spoligonogenies of ~0.1% (Table 2). The new mutants, along with the three previously identified mutants with Spo^H phenotype, were analyzed for expression of the spoIIG::lacZ promoter fusion (Fig. 1).

The strain carrying the wild-type spo0A gene showed stimulation of the spoIIG promoter beginning at 1 h after the end of log phase (T_1) and reaching a maximum at T_2. Deletion of either the 10 or the 15 C-terminal amino acids of Spo0A (spo0AΔ10 and spo0AΔ15) resulted in a reduction of spoIIG promoter activity to 14 and 10% of the wild-type level, respectively. Mutants DR2006 (D258V) and DR2008 (L260V) and the previously known mutants DR2001 (A257V) and DR2002 (A257E) showed less than 10% of wild-type expression of the promoter, a level similar to that in a spo0A null strain (5, 12). To test whether the mutants that could not activate the
To determine the mutation (20), the D258V and L260V mutations did not activate the promoter, which carries an abrB fusion integrated into the spoIIA locus, selecting for spectinomycin resistance, which is associated with the fusion in this strain. Cultures of the transformed strains were grown and the level of spoIIA::lacZ promoter activity monitored. Since none of the valine substitutions destabilized theSpo0A protein, we tested by monitoring the activity of the abrB promoter, which is repressed by Spo0A-P (19, 20, 24, 25). The spoIIG::lacZ fusion in JH642, DM2001, DR2006, and DR2008 was replaced by transforming the strains with DNA form strain JH12604 (obtained from M. Perego, Scripps Institute), which carries an abrB::lacZ fusion integrated into the amyE locus, selecting for spectinomycin resistance, which is associated with the fusion in this strain. Cultures of the transformants were grown and the level of β-galactosidase activity was measured. The results (Fig. 2) showed that, like the A257V mutation (20), the D258V and L260V mutations did not activate the spoIIG promoter.

The possibility that the valine substitutions destabilized the Spo0A protein was tested by monitoring the activity of the abrB promoter, which is repressed by Spo0A-P (19, 20, 24, 25). The spoIIG::lacZ fusion in JH642, DM2001, DR2006, and DR2008 was replaced by transforming the strains with DNA form strain JH12604 (obtained from M. Perego, Scripps Institute), which carries an abrB::lacZ fusion integrated into the amyE locus, selecting for spectinomycin resistance, which is associated with the fusion in this strain. Cultures of the transformants were grown and the level of β-galactosidase activity was measured. The results (Fig. 2) showed that, like the A257V mutation (20), the D258V and L260V mutations did not activate the spoIIG promoter.

The possibility that the valine substitutions destabilized the Spo0A protein was tested by monitoring the activity of the abrB promoter, which is repressed by Spo0A-P (19, 20, 24, 25). The spoIIG::lacZ fusion in JH642, DM2001, DR2006, and DR2008 was replaced by transforming the strains with DNA form strain JH12604 (obtained from M. Perego, Scripps Institute), which carries an abrB::lacZ fusion integrated into the amyE locus, selecting for spectinomycin resistance, which is associated with the fusion in this strain. Cultures of the transformants were grown and the level of β-galactosidase activity was measured. The results (Fig. 2) showed that, like the A257V mutation (20), the D258V and L260V mutations did not activate the spoIIG promoter.

The possible that the valine substitutions destabilized the Spo0A protein was tested by monitoring the activity of the abrB promoter, which is repressed by Spo0A-P (19, 20, 24, 25). The spoIIG::lacZ fusion in JH642, DM2001, DR2006, and DR2008 was replaced by transforming the strains with DNA form strain JH12604 (obtained from M. Perego, Scripps Institute), which carries an abrB::lacZ fusion integrated into the amyE locus, selecting for spectinomycin resistance, which is associated with the fusion in this strain. Cultures of the transformants were grown and the level of β-galactosidase activity was measured. The results (Fig. 2) showed that, like the A257V mutation (20), the D258V and L260V mutations did not activate the spoIIG promoter.

We modified the classical alanine-scanning mutagenesis technique (26) to probe the extreme C-terminal residues of Spo0A because the target region contained several alanine residues, and one valine substitution mutation, at position 257, had already been isolated (20). Next to alanine, valine is the most suitable amino acid for negating electrostatic effects while minimizing additional steric effects. Four of the 10 C-terminal amino acids of Spo0A have positively charged side chains. Since none of the valine substitutions at these residues affected Spo0A activity, we concluded that the C terminus was not a "positive charge patch" needed for transcription activation. The A257V, D258V, and L260V mutations affected both σH- and σE-dependent transcription activation. The isolation of two intragenic suppressors of A257V (20), H162R (swt4) and L174F (swt3), suggests that A257, and, by extension, D258 and L260 could be involved in maintaining the activated structure.

### Table 1. Amino acid sequences of C terminus mutants of Spo0A

| Protein          | Amino acid sequence | Reference or source |
|------------------|--------------------|---------------------|
| Spo0A (WT)       | EFIAMVADKLRLHES    | 9                   |
| Spo0AA10         | EFIAMVAA           | This study          |
| Spo0AA15         | EF                | 9                   |
| Spo0AA275V (spo0AA9V) | EFIAMVADKLRLHES   | 9                   |
| Spo0AA257E (spo0AA153) | EFIAMVADKLRLHES  | This study          |
| Spo0AD258V       | EFIAMVADKLRLHES    | This study          |
| Spo0AK259V       | EFIAMVADKLRLHES    | This study          |
| Spo0AL260V       | EFIAMVADKLRLHES    | This study          |
| Spo0AR261V       | EFIAMVADKLRLHES    | This study          |
| Spo0AS267V       | EFIAMVADKLRLHES    | This study          |
| Spo0AS266V       | EFIAMVADKLRLHES    | This study          |
| Spo0AS265V       | EFIAMVADKLRLHES    | This study          |
| Spo0AS264V       | EFIAMVADKLRLHES    | This study          |

* The position of the substitution is underlined.
* WT, wild type.

### Table 2. Effects of Spo0A mutations on spore formation

| Strain   | Protein | No. of cells/ml | No. of spores/ml | Sporulation frequency (%) |
|----------|---------|-----------------|------------------|---------------------------|
| JH16304  | Spo0A   | 4.0 x 10^6      | 2.8 x 10^6       | 70                        |
| DR2001   | Spo0A   | 4.0 x 10^6      | 4.0 x 10^4       | <0.1                      |
| DR2002   | Spo0A   | 2.0 x 10^4      | <1               | <0.1                      |
| DR2004   | Spo0A   | 4.0 x 10^4      | 100              | <0.1                      |
| DR2005   | Spo0A   | 2.2 x 10^7      | <1               | <0.1                      |
| DR2006   | Spo0A   | 4.0 x 10^6      | <1               | <0.1                      |
| DR2007   | Spo0A   | 4.0 x 10^6      | 3.0 x 10^8       | 75                        |
| DR2008   | Spo0A   | 2.0 x 10^7      | 5.0 x 10^2       | <0.1                      |
| DR2009   | Spo0A   | 4.0 x 10^7      | 4.0 x 10^8       | 100                       |
| DR2010   | Spo0A   | 5.0 x 10^7      | 3.0 x 10^8       | 60                        |
| DR2011   | Spo0A   | 5.0 x 10^6      | 4.0 x 10^4       | 80                        |
| DR2012   | Spo0A   | 3.7 x 10^6      | 3.0 x 10^8       | 81                        |
| DR2013   | Spo0A   | 5.0 x 10^6      | 3.0 x 10^8       | 60                        |
| DR2014   | Spo0A   | 3.8 x 10^6      | 2.7 x 10^8       | 71                        |
| DR2015   | Spo0A   | 5.0 x 10^6      | 1.5 x 10^6       | 30                        |

* Cells were grown in Schaeffer sporulation medium (14) and sporulation frequency was determined as described in reference 6.
of Spo0A. A similar role has been assigned to the residues in the extreme C terminus of OmpR, which interacts with central amino acids to create a compact hydrophobic structure (16).

Loss of spoIIA activation in the A257V mutant has been interpreted as an indication that this region is needed for specific interaction with the σH subunit of RNA polymerase (20). The hypothesis that Spo0A-P contacts σH and σA with different subdomains is attractive, since mutations in the σA contact region do not affect activation of σH-dependent promoters (4, 12, 14, 22). However, while σA-independent promoters are known that reduce transcription from Spo0A-dependent but not Spo0A-independent promoters (2, 4, 22), no Spo0A mutants are known that block activation of σA-dependent promoters but not σH-dependent promoters. Furthermore, the available data suggest that the Spo0A binding sites (0A boxes) that are critical for activation of the spoIIA promoter are located further upstream than are the 0A boxes needed for spoIIG activation, and they also suggest that the orientation of the 0A boxes upstream of the spoIIA promoter is inverted relative to the orientation of the 0A boxes upstream of the spoIIG promoter (23, 27). These factors lead to the possibility that the mechanism of Spo0A activation at σH-dependent promoters is different than the mechanism at σA-dependent promoters. The mutations identified in this study are consistent with this view, although a more general role for these residues in maintaining the structure of the protein cannot be ruled out.

We thank M. Cervin for comments on the manuscript and J. A. Hoch and M. Perego for providing strains. This work was supported by grants from the National Science and Engineering Research Council of Canada and the Medical Research Council of Canada to G.B.S.

REFERENCES
1. Baldus, J. M., B. D. Green, P. Youngman, and C. P. Moran, Jr. 1994. Phosphorylation of Bacillus subtilis transcription factor Spo0A stimulates transcription from the spoIIG promoter by enhancing binding to weak 0A boxes. J. Bacteriol. 176:290–306.
2. Baldus, J. M., C. M. Buckner, and C. P. Moran, Jr. 1995. Evidence that the transcriptional activator Spo0A interacts with two sigma factors in Bacillus subtilis. Mol. Microbiol. 17:281–290.
3. Brehm, S. P., S. P. Staal, and J. A. Hoch. 1973. Phenotypes of pleiotropic-negative sporulation mutants of Bacillus subtilis. J. Bacteriol. 115:1063–1070.
4. Buckner, C. M., and C. P. Moran, Jr. 1998. A region in Bacillus subtilis σH required for Spo0A-dependent promoter activity. J. Bacteriol. 180:4987–4990.
5. Buckner, C. M., G. Schyns, and C. P. Moran, Jr. 1998. A region in the Bacillus subtilis transcription factor Spo0A that is important for spoIIG promoter activation. J. Bacteriol. 180:3578–3583.
6. Dartois, V., T. Djavakhishvili, and J. A. Hoch. 1996. Identification of a membrane protein involved in activation of the KinB pathway to sporulation in Bacillus subtilis. J. Bacteriol. 178:1178–1186.
7. Errington, J. 1993. Bacillus subtilis sporulation: regulation of gene expression and control of morphogenesis. Microbiol. Rev. 57:1–33.
8. Ferrari, E., S. M. H. Howard, and J. A. Hoch. 1986. Effect of stage 0 sporulation mutations on subtilisin expression. J. Bacteriol. 166:171–176.
9. Ferrari, F. A., K. Truch, D. LeCog, J. Spence, E. Ferrari, and J. A. Hoch. 1985. Characterization of the spo0A locus and its deduced product. Proc. Natl. Acad. Sci. USA 82:2647–2651.
10. Greene, E. A., and G. B. Spiegelman. 1996. The Spo0A protein of Bacillus subtilis inhibits transcription of the abrB gene without preventing binding of the polymerase to the promoter. J. Biol. Chem. 271:11455–11461.
11. Grimsley, J. K., R. B. Tjalkens, M. A. Strauch, T. H. Bird, G. B. Spiegelman, Z. Hostomsky, J. M. Whiteley, and J. A. Hoch. 1996. Subunit composition and domain structure of the Spo0A sporulation transcription factor of Bacillus subtilis. J. Biol. Chem. 271:16977–16982.
12. Hatt, J. K., and P. Youngman. 1998. Spo0A mutants of Bacillus subtilis with sigma factor-specific defects in transcription activation. J. Bacteriol. 180:3584–3591.
13. Higed, T. B., J. A. Hoch, and J. Spizen. 1972. Hyperprotease-producing mutants of Bacillus subtilis. J. Bacteriol. 112:1026–1028.
14. Hoch, J. A. 1991. Genetic analysis in Bacillus subtilis. Methods Enzymol. 204:305–320.
15. Hoch, J. A. 1993. Regulation of the phosphorelay and the initiation of sporulation in Bacillus subtilis. Annu. Rev. Microbiol. 47:441–466.
16. Kondo, H., A. Nakagawa, J. Nishihira, Y. Nishimura, T. Mizuno, and T. Tanaka. 1997. Escherichia coli positive regulator OmpR has a large loop structure at the putative RNA polymerase interaction site. Nat. Struct. Biol. 4:28–31.
17. Kudoh, J., T. Ikekuchi, and K. Kurahashi. 1985. Nucleotide sequences of the sporulation gene spo0A and its mutant genes of Bacillus subtilis. Proc. Natl. Acad. Sci. USA 82:2665–2668.
18. Perego, M. 1993. Integration vectors for genetic manipulation in Bacillus subtilis. In J. L. Sommehien, J. A. Hoch, and R. Losick (ed.), Bacillus subtilis and other gram-positive bacteria. American Society for Microbiology, Washington, D.C.
19. Perego, M., G. B. Spiegelman, and J. A. Hoch. 1988. Structure of the gene for the transition state regulator, AbrB: regulator synthesis is controlled by the spo0A sporulation gene in Bacillus subtilis. Mol. Microbiol. 2:689–699.

20. Perego, M., J.-J. Wu, G. B. Spiegelman, and J. A. Hoch. 1991. Mutational dissociation of the positive and negative regulatory properties of the Spo0A sporulation transcription factor of Bacillus subtilis. Gene 100:207–212.

21. Rowe-Magnus, D. A. 1998. The mechanism of transcription activation by the Bacillus subtilis response regulator, Spo0A. Ph.D. thesis. University of British Columbia, Vancouver, British Columbia, Canada.

22. Schyns, G., C. M. Buckner, and C. P. Moran, Jr. 1997. Activation of the Bacillus subtilis spoIIG promoter requires interaction of Spo0A and the sigma subunit of RNA polymerase. J. Bacteriol. 179:5605–5608.

23. Spiegelman, G. B., T. H. Bird, and V. Voon. 1995. Transcription regulation by the Bacillus subtilis response regulator Spo0A, p. 159–179. In J. A. Hoch and T. J. Silhavy (ed.), Two-component signal transduction. ASM Press, Washington, D.C.

24. Strauch, M., V. Webb, G. B. Spiegelman, and J. A. Hoch. 1990. The Spo0A protein of Bacillus subtilis is a repressor of the abrB gene. Proc. Natl. Acad. Sci. USA 87:1801–1805.

25. Strauch, M. A., and J. A. Hoch. 1993. Transition-state regulators: sentinels of Bacillus subtilis post-exponential phase gene expression. Mol. Microbiol. 7:337–342.

26. Wells, J. A. 1991. Systematic mutational analysis of protein-protein interfaces. Methods Enzymol. 202:390–411.

27. Wu, J.-J., P. J. Piggot, K. M. Tatti, and C. P. Moran, Jr. 1991. Transcription of the Bacillus subtilis spoIIG locus. Gene 101:113–116.