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Spo0A Mutants of *Bacillus subtilis* with Sigma Factor-Specific Defects in Transcription Activation

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The transcription factor Spo0A of *Bacillus subtilis* has the unique ability to activate transcription from promoters that require different forms of RNA polymerase holoenzyme. One class of Spo0A-activated promoter, which includes *spoIIEp*, is recognized by RNA polymerase associated with the primary sigma factor, sigma A (σ^A); the second, which includes *spoIIAp*, is recognized by RNA polymerase associated with an early-sporulation sigma factor, sigma H (σ^H). Evidence suggests that Spo0A probably interacts directly with RNA polymerase to activate transcription from these promoters. To identify residues of Spo0A that may be involved in transcriptional activation, we used PCR mutagenesis of the entire *spo0A* gene and designed a screen using two distinguishable reporter fusions, *spoIIE-gus* and *spoIIA-lacZ*. Here we report the identification and characterization of five mutants of Spo0A that are specifically defective in activation of σ^A-dependent promoters while maintaining activation of σ^H-dependent promoters. These five mutants identify a 14-amino-acid segment of Spo0A, from residue 227 to residue 240, that is required for transcriptional activation of σ^A-dependent promoters. This region may define a surface or domain of Spo0A that makes direct contacts with σ^A-associated holoenzyme.

The phosphorylation-activated transcription factor Spo0A of *Bacillus subtilis* is a member of the response regulator family of two-component signal transduction proteins that regulates the initiation of sporulation (1, 27, 34). Under conditions of nutrient limitation, extracellular and intracellular signals are processed through a complex signal transduction pathway that controls the phosphorylation state of Spo0A (14, 16, 17). Phosphorylation of Spo0A increases its affinity for a 7-bp consensus DNA sequence, 5′-TGTCGAA-3′, referred to as the 0A box (5, 35). Spo0A binding then serves either to repress transcription of genes such as *abrB*, which encodes a regulator protein required for the transition into stationary phase (35), or to activate transcription of various sporulation-specific genes, such as *spoIIA* (9, 37), *spoIIE* (41), and *spoIIG* (32). Although much has been learned about the signal transduction network that controls the phosphorylation state of Spo0A, little is known about the mechanism by which Spo0A stimulates transcription from promoters under its control.

Spo0A is unique in its ability to activate transcription from promoters that require different forms of RNA polymerase (RNAP) holoenzyme for transcription. The *spoIIA* promoter is Spo0A dependent and is recognized by RNAP associated with sigma H (σ^H) (38, 39), an early-sporulation sigma factor (11). The *spoIIE* and *spoIIG* promoters are also Spo0A dependent but are recognized by RNAP associated with sigma A (σ^A) (5, 31, 41), the primary sigma factor of *B. subtilis*. Genetic and biochemical evidence indicates that Spo0A binds to these promoters at multiple sites (5, 31, 41) and that, upon phosphorylation, it binds with increased affinity to certain sites to activate transcription (5).

In *Escherichia coli*, two major classes of transcriptional activators have been identified: the class I and class II activators (10, 15, 18). Class I activators are characterized by DNA binding sites upstream of the −35 region of the promoter (18). Evidence suggests that class I activators make direct contacts to the alpha subunit of RNAP to activate transcription (18). In contrast, class II activators promote transcription by binding at or near the −35 region of the promoter and appear to make direct contact with the sigma subunit of RNAP (18, 21, 25). The promoters, *spoIIAp*, *spoIIEp*, and *spoIIGp*, positively regulated by Spo0A have been characterized in detail, and each contains a Spo0A-binding site in its −35 region (5, 31, 32, 37, 41); in some cases, these binding sites have been demonstrated to be of functional importance in vitro and/or in vivo (5, 12, 31, 32, 37, 39, 41). Thus, there was an expectation that Spo0A might conform to the pattern observed for class II activators and that its mechanism of action might involve direct interaction with the sigma subunit of RNAP holoenzyme. This inference was supported by the identification of mutations in both the σ^A and σ^H factors of *B. subtilis* that impair expression of Spo0A-dependent promoters but not of Spo0A-independent promoters (4, 33).

If Spo0A does stimulate transcription through direct interaction with σ, it is interesting to consider whether Spo0A contacts σ^A and σ^H in the same way. We have addressed this question in the present work by asking whether it is possible to isolate mutants of Spo0A that show sigma-specific defects. We report the characterization of five such mutants. In each case, the mutants show a drastic reduction in the ability to stimulate transcription of σ^A-dependent promoters while retaining nearly wild-type ability to stimulate σ^H-dependent promoters. Interestingly, the five mutations that cause this phenotype are clustered in a 14-amino acid (aa) segment of the protein. We speculate that this segment may represent a surface or domain of Spo0A that interacts directly with the σ subunit of σ^A-associated RNAP holoenzyme. No representatives of the reciprocal class of mutant, in which Spo0A-dependent promoters that utilize σ^H-associated holoenzyme were specifically affected, were detected. This may indicate mechanistic differences in the way the two holoenzyme forms are influenced by Spo0A, although other explanations are considered.

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TABLE 1. Bacterial strains used in this work

Strain	Genotype or description	Source or reference
E. coli MM294	*endA1 hsdR17 supE44 thi-1*	3
B. subtilis		
PY79	Prototroph	43
JKH63	PY79 *spoIIE*::pJKH9	This work
JKH68	PY79 *spoIIA*::pPP81	This work
JKH72	PY79 *spoIIA*::pPP81 *spoIIE*::pJKH9	This work
JKH73	JKH72 *spo0A*::pSPC101-0A	This work
JKH74	JKH72 *spo0A*::pSPC101-G227R	This work
JKH75	JKH72 *spo0A*::pSPC101	This work
JKH104	JKH72 *spo0A*::pSPC101-S233P	This work
JKH105	JKH72 *spo0A*::pSPC101-F236S	This work
JKH106	JKH72 *spo0A*::pSPC101-V240A	This work
JKH107	JKH72 *spo0A*::pSPC101-V240G K265R	This work
JKH111	PY79 *spoIIG*::IIG-lacZ *spo0A*::pSPC101-0A	This work
JKH112	PY79 *spoIIG*::IIG-lacZ *spo0A*::pSPC101	This work
JKH114	PY79 *spoIIG*::IIG-lacZ *spo0A*::pSPC101-G227R	This work
JKH115	PY79 *spoIIG*::IIG-lacZ *spo0A*::pSPC101-F236S	This work
JKH116	PY79 *spoIIG*::IIG-lacZ *spo0A*::pSPC101-V240A	This work
JKH117	PY79 *spoIIG*::IIG-lacZ *spo0A*::pSPC101-S233P	This work
JKH118	PY79 *spoIIG*::IIG-lacZ *spo0A*::pSPC101-V240G K265R	This work

MATERIALS AND METHODS

Bacterial strains, culture media, genetic techniques, and in vitro manipulation of DNA. Bacterial strains used in this work are listed in Table 1. Routine microbiological procedures and enzymatic manipulations of DNA were carried out by standard methods (2, 13). The concentrations of antibiotics used for selection on Luria-Bertani (LB) or Difco sporulation medium (DSM) agar and in culture were 5 μg/ml for chloramphenicol, 3 μg/ml for neomycin, 100 μg/ml for spectinomycin, and 100 μg/ml for ampicillin. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) (both from U.S. Biological) in dimethyl sulfoxide were used at a final concentration of 75 μg/ml in indicator agar.

Construction of a *B. subtilis* screening strain containing two promoter fusions. A *spoIIA-lacZ* promoter fusion strain was generated by transformation of *B. subtilis* PY79 to chloramphenicol resistance by integration of the vector pPP81 (30) into the *B. subtilis* chromosome. This strain was then transformed to neomycin resistance with chromosomal DNA isolated from a *spoIIE-gus* promoter fusion strain. The *spoIIE-gus* fusion strain was constructed by integration of the clone pJKH9 into the *B. subtilis* chromosome. This clone was generated by first cloning a 275-bp *Hin*dIII-*Bam*HI fragment containing the *spoIIE* promoter from pGV49 (12) into the pBluescript polylinker (Stratagene), then subsequently cloning a 290-bp *Sal*I-*Bam*HI fragment containing the promoter into the vector pMLK117 (19). The pMLK117 vector contained a promoterless copy of the *gus* gene, a neomycin resistance marker selectable in a single copy in *B. subtilis*, unique sites for the cloning of promoters upstream of the *gus* gene, and an origin of replication and a selectable marker functional in *E. coli*. The resulting screening strain, JKH72, carried a *spoIIA-lacZ* transcriptional fusion and a *spoIIE-gus* transcriptional fusion and was chloramphenicol resistant, neomycin resistant, and sporulation proficient (Spo$^+$) due to restoration of intact copies of the two fused genes, *spoIIA* and *spoIIE*.

Construction of the pSPC101 integrational vector. A 1.2-kb blunt-ended fragment carrying the spectinomycin resistance gene from *Enterococcus faecalis* (22) was cloned by ligation into the *Sma*I site of pUC19 (40). A 2.2-kb *Eco*RI fragment from pJRS233 (29) containing the pSC101 origin of replication was then cloned into the *Eco*RI site of the pUC plasmid carrying the spectinomycin resistance marker. The resulting 6.1-kb plasmid was digested with *Pvu*II, resulting in two fragments of 3.7 and 2.4 kb. The 3.7-kb fragment was then gel purified and recircularized to form the pSPC101 integrational vector. The key features of the vector are the pSC101 origin of replication functional in *E. coli*, the spectinomycin resistance marker from *E. faecalis* selectable in both *E. coli* and *B. subtilis*, and a portion of the pUC19 polylinker containing unique cloning sites (Fig. 1A). The use of this vector decreased the possibility of homologous recombination into already existing pUC vector sequences in the chromosome of the screening strain.

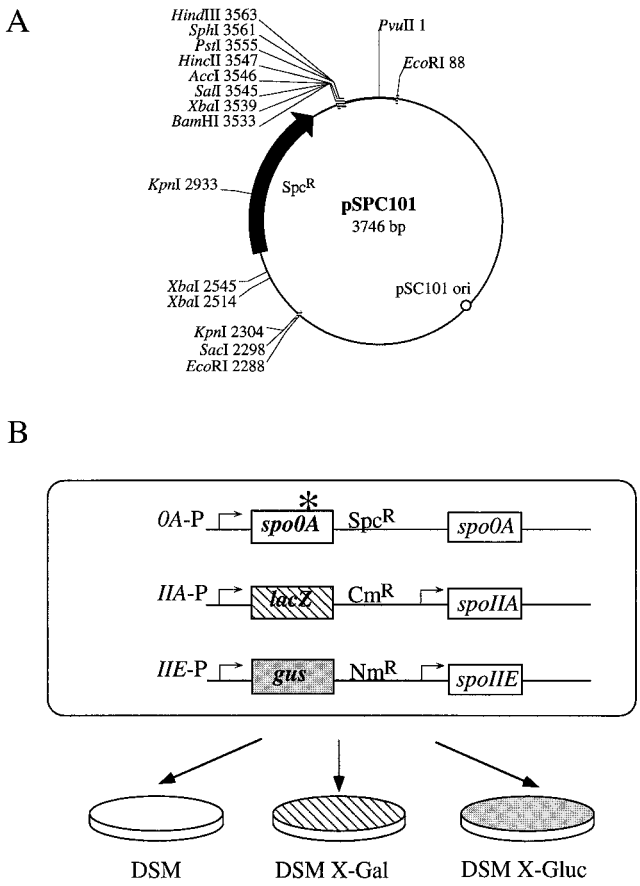

FIG. 1. Key components of the mutant screen. (A) Restriction map of the integrational vector pSPC101 used to introduce mutagenized *spo0A* into the screening strain. Spc^R, spectinomycin resistance; pSC101 ori, origin of replication functional in *E. coli*. (B) Schematic representation of the screen. The screening strain JKH72 contains a *spoIIA-lacZ* fusion as a reporter of Spo0A-dependent, σ^H-dependent transcription activity and a *spoIIE-gus* fusion as a reporter of Spo0A-dependent, σ^A-dependent transcription activity. Transcription activation of these two reporter fusions is screened on X-Gal and X-Gluc indicator agars. The sporulation phenotype is screened on DSM agar. See Materials and Methods for a detailed description of the screen.

Construction of isogenic *spo0A* wild-type (JKH73) and null (JKH75) control strains. The *spo0A* gene was amplified by PCR from the chromosome of a wild-type strain of *B. subtilis*. The primers used to amplify the gene were 0Aup*Bam*HI (5′-GCAGTAGGATCCATGTAGCAAGGGTGAATCC-3′) and 0A4*Hin*dIII (5′-GCAGGAAGCTTCGCCTCCTATTTATCAGCGC-3′), which incorporate *Bam*HI and *Hin*dIII restriction sites, respectively, at the ends of the PCR product to facilitate cloning into the pSPC101 integrational vector. This construct was used to transform the screening strain JKH72 by selecting for spectinomycin resistance. Integration at the *spo0A* locus was confirmed by PCR, and the wild-type sequence was verified by sequencing the allele on both strands with the *fmol* sequencing kit (Promega). The JKH73 strain was blue both on X-Gal agar after 20 h of incubation at 37°C and on X-Gluc agar after 48 h of incubation at 37°C when screened for expression of the two promoter fusions carried in the strain. This strain was Spo$^+$ when screened on DSM plates after 48 h of incubation at 37°C. Sporulation phenotypes were assessed directly on DSM agar by the formation of brown pigment by Spo$^+$ strains (26). A null allele of *spo0A* was generated by cloning an internal 420-bp *Sau*IIIA fragment of the *spo0A* coding region into the *Bam*HI site of pSPC101. The integration of this clone into the chromosomal *spo0A* locus disrupted the coding region of the *spo0A* gene, resulting in a null strain, JKH75. Integration at the *spo0A* locus was confirmed by PCR. JKH75 was white on both X-Gal agar and X-Gluc agar and had a Spo$^-$ phenotype when screened on DSM agar.

Mutagenesis of *spo0A*. Random mutations were introduced into the *spo0A* gene by PCR amplification of the coding sequence under conditions previously

described (23). The reaction differed from this protocol by the omission of dimethyl sulfoxide and β-mercaptoethanol and the use of either 0.5 mM $MnCl_2$ alone or 0.25 mM $MnCl_2$ and a 5:1 ratio of dGTP to dATP. The primers 0Aup*Bam*HI and 0A4*Hind*III used for the amplification are described above. *Bam*HI and *Hin*dIII restriction sites were used to clone the mutagenized products into the pSPC101 vector.

The screen for Spo0A mutants defective in transcription activation. The *spoIIA-lacZ* fusion was used as a reporter of Spo0A-dependent, σ^H-dependent gene expression, and the *spoIIE-gus* fusion was used as a reporter of Spo0A-dependent, σ^A-dependent gene expression. PCR-mutagenized *spo0A* genes were cloned into the pSPC101 integrational vector, and this pool of mutagenized *spo0A* clones (pSPC101-0A*) was then used to transform *E. coli* MM294 to spectinomycin resistance. Transformants were pooled, and plasmid DNA was isolated. The plasmid DNA was restricted at a unique *Sac*I site and ligated together to form multimers in order to facilitate plasmid integration into the *B. subtilis* chromosome. JKH72 was transformed by pSPC101-0A* DNA, and integrants were selected by plating to LB or DSM agar containing spectinomycin (Fig. 1B). Expression of the *spoIIA-lacZ* fusion in strains transformed by pSPC101-0A* was determined by picking transformants to DSM agar containing chloramphenicol, neomycin, spectinomycin, and X-Gal. Expression of the *spoIIE-gus* fusion was determined by picking the same transformants to DSM agar containing the same antibiotics and X-Gluc. The ability to sporulate was determined by patching to DSM plates containing the appropriate antibiotics. Expression of the promoter fusions was determined by the presence or absence of blue color on the indicator plates after 20 h of incubation at 37°C for X-Gal and 48 h of incubation at 37°C for X-Gluc. Blue color indicated the expression of the fusion product.

Sequencing of mutant *spo0A* alleles. The *spo0A* gene of *spo0A225* was amplified from the chromosome of the mutant strain by PCR using primers which amplify from a site in the promoter region of *spo0A* (0A71R, 5′-TCTTCACTT CTCAGAATACATACGG-3′) and from a site downstream of the gene (0A1190L, 5′-ACAAATGTCCCCAAAACAAAACGCC-3′). The *spo0A* alleles of the other mutant strains were amplified from chromosomal DNA by PCR using the same upstream primer (0A71R) and a primer which anneals to a site in the integrated plasmid vector (pUC reverse primer 1224, 5′-GCCAGGGTT TTCCCAGTCACGAC-3′). The PCR products were purified from the reaction mixture by using the Wizard PCR purification kit and were directly sequenced by using the *fmol* sequencing kit (both from Promega). Sequencing reactions were carried out on both strands of the PCR products.

β-Galactosidase and β-glucuronidase assays. β-Galactosidase assays were performed by the fluorometric method of Youngman (42). Control and mutant *spo0A* strains containing both a *spoIIA-lacZ* transcriptional fusion and a *spoIIE-gus* transcriptional fusion were streaked to LB agar containing chloramphenicol, neomycin, and spectinomycin and were incubated overnight at 30°C to produce a very light lawn of growth. Bacteria were washed from the plates with LB medium and were used to directly inoculate 5 ml of LB containing chloramphenicol, neomycin, and spectinomycin to barely detectable turbidity. The 5-ml cultures were allowed to resume growth to mid-log phase and were used to inoculate 30 ml of DSM to a reading of ≤5 Klett units. Bacteria were cultured for assay at 37°C with shaking. At various intervals during growth and sporulation, 0.5-ml samples were collected and frozen in liquid nitrogen. Samples were stored at −70°C until assayed.

β-Glucuronidase assays were performed in an identical manner, except that 0.4 mg of 4-methylumbelliferyl-β-D-glucuronide trihydrate (MUG) substrate (U.S. Biological)/ml specific to β-glucuronidase was used instead of 0.4 mg of 4-methylumbelliferyl-β-D-galactoside (U.S. Biological)/ml. One unit of activity is defined as one picomole of MUG hydrolyzed per milliliter of culture sample per minute, normalized for culture cell density (turbidity). Each sample was assayed for both β-glucuronidase and β-galactosidase activities.

Wild-type and mutant *spo0A* strains containing a *spoIIG-lacZ* transcriptional fusion were grown and samples for assay were collected in the manner described above except that the medium used for growth contained chloramphenicol and spectinomycin. β-Galactosidase assays were performed as described above.

Sporulation frequency assay. *B. subtilis* strains were grown for 48 h at 37°C on DSM agar containing the appropriate antibiotics. A single colony was resuspended in 0.5 ml of DSM. The number of viable cells was determined by dilution and plating onto LB agar containing the appropriate antibiotics. The number of heat-resistant spores was determined by heating the resuspended cells at 80°C for 20 min and plating appropriate dilutions on selective plates. The sporulation frequency was determined as the percentage of the number of heat-resistant spores compared to the total number of viable cells before heat treatment. The sporulation frequency for each mutant was calculated as the average from three independent assays.

Immunoblot detection of Spo0A proteins. Polyclonal anti-Spo0A antibodies were raised in rabbits by using heparin agarose-purified Spo0A. Samples (10 to 25 ml) of *B. subtilis* cultures grown in DSM at 37°C with shaking were collected, and the cells were harvested at various time points. Cell pellets were washed in a buffer previously described (5), quick-frozen in an ethanol-dry ice bath, and stored at −70°C until assayed. Cell pellets were resuspended in 1 ml of buffer (5), and crude extracts were prepared. The cells were lysed at 4°C by two passages through a French pressure cell at 19,000 lb/in^2. Total protein was quantitated by using the Bio-Rad Protein microassay procedure as described by the manufacturer. Protein samples (10 and 0.625 μg) were separated by electrophoresis through a sodium dodecyl sulfate (SDS)–12% polyacrylamide gel. Proteins were electroblotted to a PVDF-Plus membrane (Micron Separations, Inc.).

The membrane was blocked by incubation for 1 h at room temperature in Tris-buffered saline–Tween (100 mM Tris · Cl [pH 8.0], 0.9% NaCl, 0.05% Tween) (TBST) containing 3% (wt/vol) bovine serum albumin (BSA). The membrane was subsequently washed three times for 15 min each time in TBST alone and then incubated with 15 ml of TBST containing 1% BSA and a 1:15,000 dilution of rabbit anti-Spo0A antiserum. After incubation for 45 min at room temperature with gentle agitation, the membrane was again washed three times for 15 min each time with TBST alone. The membrane was then incubated with 15 ml of TBST containing 1% BSA and a 1:3,000 dilution of the goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugate (Promega) for 45 min at room temperature with gentle agitation. After incubation, the membrane was washed three times for 15 min each time with TBST alone and then treated with the Renaissance Chemiluminescent Reagent (DuPont, NEN) according to the manufacturer's instructions. Treated membranes were immediately exposed to X-ray film for 10 to 30 s.

RESULTS

Mutant *spo0A225*. While screening for mutants that maintained expression of the *spoIIA* operon (which encodes σ^F) but failed to activate σ^F-controlled gene expression in the forespore, Levin and Losick (24) identified a mutation that mapped within or near the *spo0A* locus. Strains containing this mutation were strongly impaired in the expression of *spoIIE*, raising the possibility that the mutant phenotype was the result of an alteration in Spo0A that produced a promoter-specific defect in transcription activation. After confirming by genetic methods that this mutation was within the *spo0A* coding sequence (data not shown), we sequenced the entire *spo0A* gene from the mutant strain. The sequence revealed a GC-to-AT base pair substitution that resulted in a glycine-to-arginine change at residue 227 of the mutant Spo0A protein. We refer to the mutant gene as *spo0A225* and to the mutant protein as G227R. The strongly differential effect of *spo0A225* on transcription from the *spoIIE* and *spoIIA* promoters is apparent in the behavior of transcriptional fusions to these two promoters (Fig. 2).

Identification of additional mutations in *spo0A* that produce promoter-specific defects. Available evidence supports a model in which Spo0A activates transcription through direct contact with RNAP (4, 8, 33). The *spo0A225* mutation might therefore act by affecting such contacts, possibly by altering the surface or domain of Spo0A that contacts σ^A. If so, we reasoned that random mutagenesis within the *spo0A* coding sequence with a screen for a phenotype similar to that of *spo0A225* might identify additional mutations that would help to further define this surface or domain. In addition, we speculated that it might be possible to identify mutants with the reciprocal phenotype, a defect in activation of σ^H-dependent promoters but not σ^A-dependent promoters under Spo0A control.

To identify activation-defective *spo0A* mutants, we constructed a *B. subtilis* screening strain, JKH72, containing transcriptional fusions with two distinguishable reporters: a *spoIIA-lacZ* promoter fusion, used as a reporter of Spo0A-dependent, σ^H-dependent transcription, and a *spoIIE-gus* promoter fusion, used as a reporter of Spo0A-dependent, σ^A-dependent transcription. Mutations were generated by PCR amplification of the entire *spo0A* gene under conditions favoring misincorporation errors, followed by direct cloning of the amplification products into the pSPC101 integrational vector (Fig. 1A). After passage through *E. coli* MM294, plasmid DNA was pooled and used to transform JKH72. Chromosomal integrations at the *spo0A* locus were selected on medium containing spectinomycin, then transferred to DSM, DSM X-Gal, and DSM X-Gluc plates containing the appropriate antibiotics for assessment of sporulation phenotypes, *spoIIA* expression, and *spoIIE*

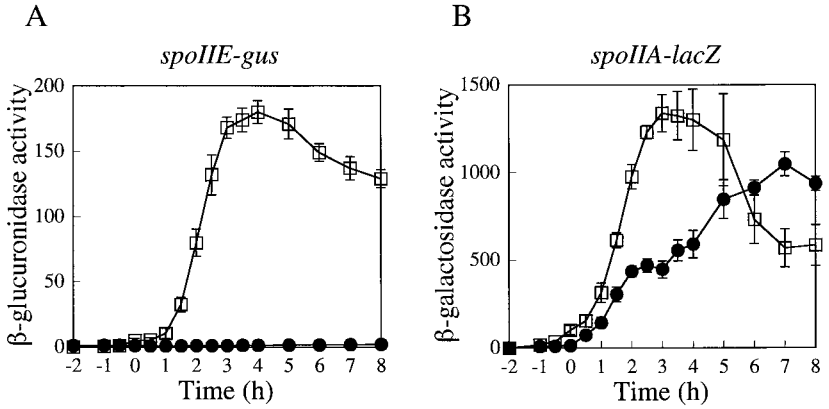

FIG. 2. Effects of the *spo0A225* mutation on transcription from two Spo0A-dependent promoters. (A) *spoIIE-gus* expression; (B) *spoIIA-lacZ* expression. Strains were cultured and assayed as described in Materials and Methods. T_0 marks the end of exponential growth. Samples were collected at 1/2-h time points from T_{-2} until T_4 and then hourly until T_8 unless otherwise indicated. Open squares, wild type (JKH73); filled circles, *spo0A225* strain. Data from at least three independent trials were averaged. Error bars, standard error of the mean (SEM). One unit of activity is defined as one picomole of MUG hydrolyzed per milliliter of culture sample per minute, normalized for culture cell density (turbidity).

expression, respectively (Fig. 1B). Because we expected that a defect in transcriptional activation of one of the stage II genes would block sporulation, the primary screen was for a Spo$^-$ phenotype. We screened 3,069 transformants and found 72 strains which were phenotypically Spo$^-$, white on X-Gluc, and blue on X-Gal, which we interpreted as indicating a defect in transcriptional activation at the *spoIIE* promoter. None of the Spo$^-$ transformants screened had the reciprocal expression pattern on the two indicator plates. Nineteen strains among the 72 mutant candidates were tested for linkage between the Spo$^-$ phenotype and spectinomycin resistance. Linkage would indicate that the phenotype observed was linked to the allele of *spo0A* present in the mutant strain. Linkage was tested by transformation of the wild-type *B. subtilis* strain PY79 to Spc^r by using chromosomal DNA from candidate Spo$^-$ strains, then assessing the number of Spo$^-$ transformants produced. Sixteen strains exhibited cotransformation frequencies greater than 90%. Five of these strains exhibiting the strongest phenotypes in our screen were chosen for further study.

Sequencing of mutant *spo0A* alleles. All five independently isolated mutant alleles of *spo0A* were sequenced through the entire structural gene, and the amino acid substitutions resulting from each mutation were determined. Remarkably, all five strains identified in the screen, like *spo0A225*, carried mutations within a 14-aa stretch in the C-terminal effector domain of the 267-aa Spo0A protein (Fig. 3). The S233P mutant was isolated as a single-base-pair substitution. As expected, however, considering the intensity of mutagenesis, most of the mutant alleles also contained substitutions at other locations within the *spo0A* gene. The S233P* mutant (the asterisk denotes a second substitution in Spo0A other than the substitution indicated) was isolated from a mutagenesis independent of that for the S233P single mutant. Each of the F236S* and V240A* mutants also contained a second substitution outside the 14-aa region. To determine whether the substitutions within the 14-aa region were responsible for the mutant phenotype, the F236S and V240A mutations were subcloned into a wild-type copy of *spo0A* in order to isolate the single substitutions and were reintroduced into JKH72. The subcloned mutants were phenotypically identical to the strains containing the double substitutions in Spo0A (data not shown). One mutant, V240G K265R, contained a second substitution near the 14-aa segment; no attempt was made to resolve whether either substitution alone might cause the mutant phenotype. The sporulation defects caused by the *spo0A* mutations are quantified in Table 2.

***spo0A* mutants that are differentially defective in activation of σ^A-dependent promoters.** Each of the mutants was tested for its ability to activate transcription from the *spoIIA* and *spoIIE* promoters. The two reporter fusions in these strains allowed us to quantitate the level of transcription from both promoters by monitoring β-galactosidase and β-glucuronidase accumulation. All five mutant strains were drastically reduced in their abilities to activate transcription of the σ^A-dependent promoter *spoIIE* (Fig. 2A and 4A) but only slightly or partially impaired at the σ^H-dependent promoter *spoIIA* (Fig. 2B and 4B). At 2 h after the start of sporulation, when the stage II gene products are expressed, *spoIIE* transcription was limited to 2.5% or less of the wild-type levels in all five mutants. These levels were similar to the activity of the promoter in a Spo0A null strain, JKH75 (<1% of wild type). All five of the mutants tested, however, showed substantial ability to activate transcription from the *spoIIA* or σ^H-dependent promoter. The S233P and V240G K265R mutants activated transcription

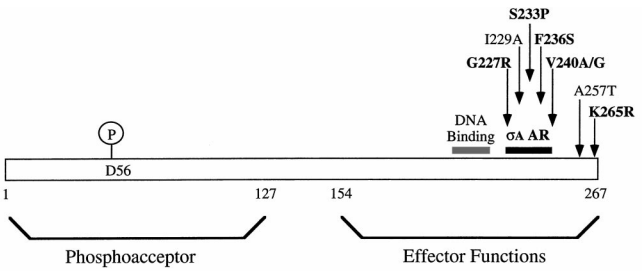

FIG. 3. Map of the activating region of Spo0A proposed for σ^A-dependent transcription. The Spo0A protein consists of two domains. The phosphoacceptor domain is formed by the N-terminal 127 aa, and the effector domain is formed by the C-terminal 113 residues; they are joined by a linker region of 27 aa, as defined by Brown et al. (7). Mutations in Spo0A protein which decrease activation of σ^A-dependent promoters under Spo0A control described in this work and elsewhere (8) are mapped. Residues examined in this study are in boldface. The σ^A activating region (σ^A AR) (solid bar) includes residues from aa 227 to aa 240. The K265 residue may also have some effect on transcription activation of σ^A-dependent promoters. Also indicated is position A257, which, when mutated, generates the *spo0A9V* mutant, which does not activate transcription of σ^H-dependent promoters (28). Shaded bar, putative DNA binding domain (aa 194 to 224) proposed for Spo0A based on sequence conservation of this region in *spo0A* homologs from diverse *Bacillus* and *Clostridium* species (7).

TABLE 2. Sporulation frequencies

Strain	Allele	% Sporulation[a]
JKH73	wt[b]	88.0
JKH75	Null[c]	0.006
JKH74	G227R	0.024
JKH104	S233P	0.141
JKH105	F236S	0.526
JKH106	V240A	0.0008
JKH107	V240G K265R	0.0002

[a] Sporulation frequencies were calculated as described in Materials and Methods.
[b] wt, wild type.
[c] Inactivation of *spo0A* generated by Campbell integration (*spo0A*::pSPC101).

from the *spoIIA* promoter only slightly less efficiently than the wild-type Spo0A strain, JKH73. The G227R and V240A mutants each showed approximately a twofold decrease in ability to activate transcription from this promoter. For the mutant with the least ability to activate transcription from the *spoIIA* promoter, F236S, transcription was approximately 4-fold less than that for the wild-type strain, JKH73, but 10-fold greater than that for the Spo0A null strain, JKH75. Because transcriptional activation at *spoIIA* is dependent on Spo0A (30, 37, 38), these results suggested that the Spo0A protein in the mutants retained the ability to bind to 0A box sites and activate transcription from the *spoIIA* promoter.

To establish whether the effect on *spoIIE* transcription was due to a general defect in utilization of σ^A-dependent promoters under Spo0A control, we transformed each of the mutant *spo0A* alleles into a *spoIIG-lacZ* fusion strain. The *spoIIG* promoter is a second σ^A-dependent promoter that is also dependent on Spo0A for activation (20). Transcriptional activation of this promoter was severely decreased (Fig. 5). At 2 h after the onset of sporulation, levels of activity at the *spoIIG* promoter in our mutant stains were indistinguishable from that of the null strain, JKH75 (approximately 11% of the wild-type level).

Spo0A protein levels in wild-type and mutant cell extracts. Although some of the *spo0A* mutants retained nearly wild-type levels of *spoIIA* expression, some reduction was detectable in all mutant strains, and the F236S mutant retained only 25% of the wild-type level of expression. To determine whether any portion of the reduction in *spoIIA* transcription in the mutants might be attributable to a decrease in Spo0A protein levels, we examined Western blots prepared with mutant and wild-type extracts, probed with polyclonal antibody to Spo0A. The results indicated slight reductions in the Spo0A protein levels of the mutants, ranging from two- to fourfold at most (Fig. 6).

DISCUSSION

The results reported here and in complementary work in the accompanying paper of Buckner et al. (8) strongly implicate a 14-aa segment of Spo0A, extending from G227 to V240 (the σ^A activating region in Fig. 3), as a region of the protein critical for transcription at promoters positively regulated by Spo0A. Motivated initially by the existence of a single mutation at G227 that appeared to cause a sigma-specific defect (24), we have subjected the entire *spo0A* coding sequence to intensive, random mutagenesis by PCR and have characterized four ad-

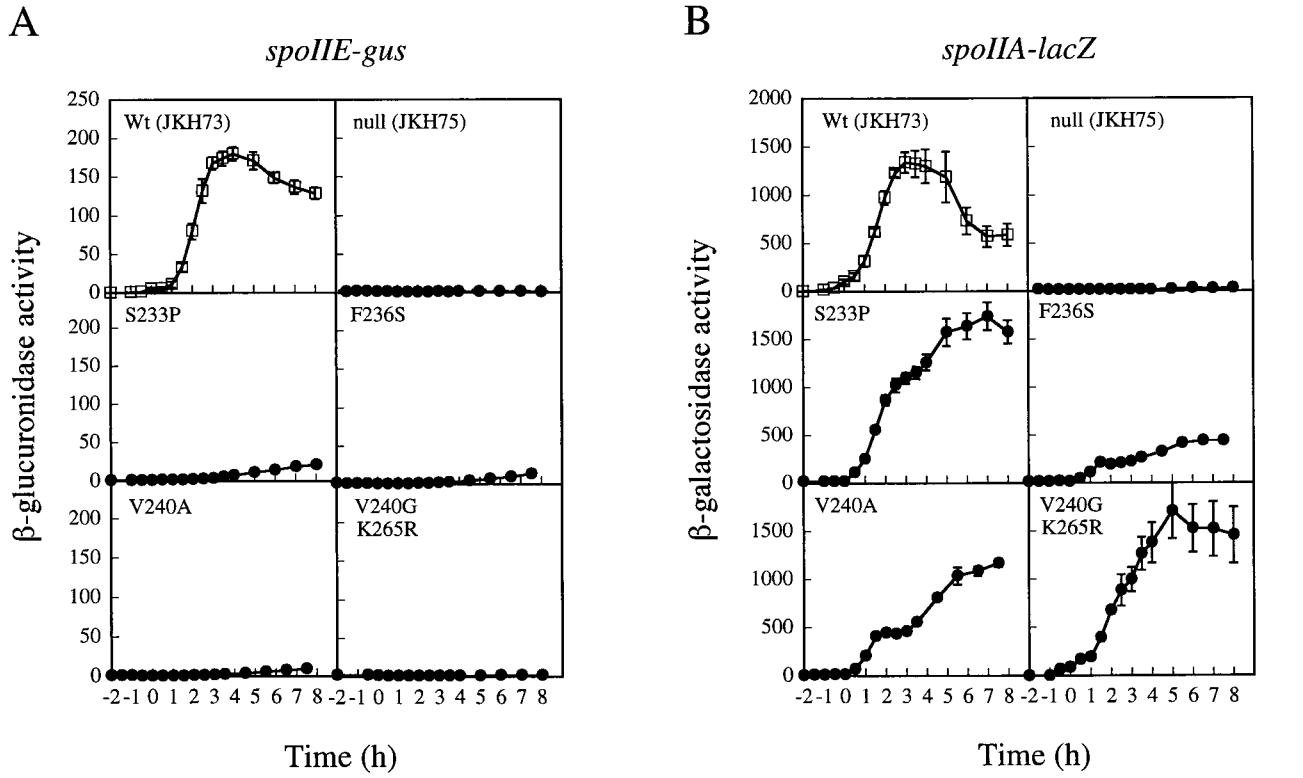

FIG. 4. Effects of *spo0A* mutants on the expression of *spoIIE-gus* (A) and *spoIIA-lacZ* (B) transcriptional fusions. Samples were collected and assayed as described in the legend to Fig. 2. In each panel, the filled circles represent the mutant indicated. Open squares represent the wild type (JKH73). Data were averaged from at least three independent trials. Error bars, SEM. One unit of activity is defined as described in the legend to Fig. 2.

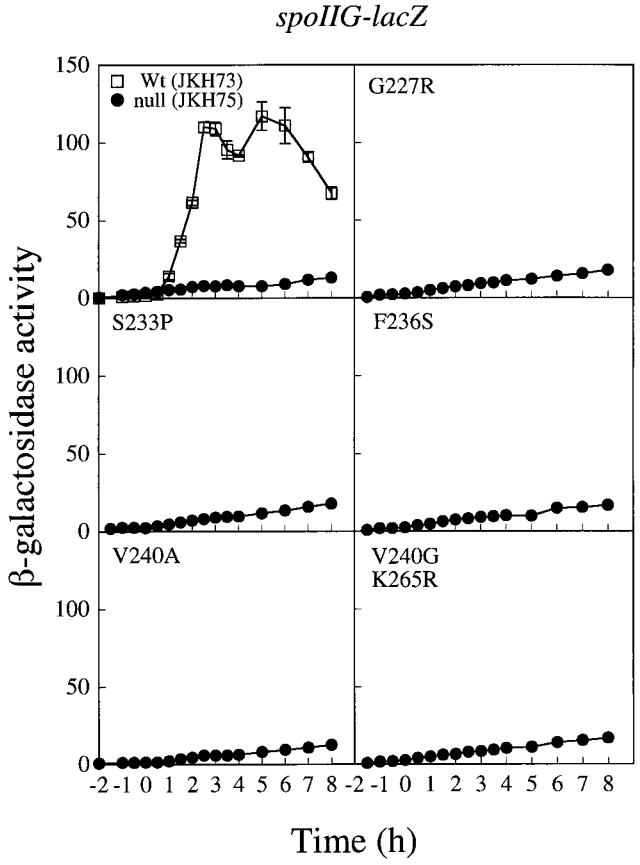

FIG. 5. Verification of a defect in transcriptional activation by *spo0A* mutants of σ^A-dependent promoters. The effect of the *spo0A* mutants on the expression of a *spoIIG-lacZ* transcriptional fusion is shown. Samples were collected and assayed as described in the legend to Fig. 2. In each panel, the filled circles represent the mutant indicated. Open squares represent the wild type (JKH73). Data were averaged from three independent trials. Error bars, SEM. One unit of activity is defined as described in the legend to Fig. 2.

ditional mutants exhibiting a similar phenotype. In all cases, the phenotype was caused by mutational changes within the 14-aa segment. The work of Buckner et al. (8) converged on precisely the same small segment of the *spo0A* coding sequence from a completely independent direction. In that study, the investigators started with a mutation in *sigA* (σ^A H359R) that specifically affected transcription from Spo0A-dependent promoters, which therefore caused a Spo$^-$ phenotype, and sought

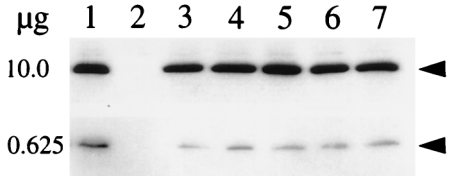

FIG. 6. Immunoblot analyses of wild-type and mutant Spo0A proteins. Culture samples were collected and harvested at 1 h after the end of exponential growth (T_1). Samples containing 10 and 0.625 μg of total cellular protein were subjected to electrophoresis through an SDS–12% polyacrylamide gel. Samples were electroblotted to a PVDF-Plus membrane and the Spo0A protein was probed with anti-Spo0A antibody as described in Materials and Methods. Lane 1 in each panel contains wild-type Spo0A (JKH73), and lanes 2 through 7 contain the following Spo0A mutants: null (JKH75) (lane 2), G227R (lane 3), S233P (lane 4), F236S (lane 5), V240A (lane 6), and V240G K265R (lane 7). Arrowheads indicate Spo0A.

a phenotypic suppressor after random localized mutagenesis (8). The suppressor they recovered (Spo0A S231F) fell within the 14-aa segment identified in our study. This segment is immediately adjacent to the region of Spo0A (residues 194 to 224) proposed to comprise the helix-turn-helix motif responsible for binding to Spo0A recognition sites in DNA (7). Taken together, our results and those reported by Buckner et al. (8) strongly support a model in which Spo0A stimulates transcription through direct contact with the σ subunit of RNAP holoenzyme, involving a surface or domain of Spo0A that includes residues 227 to 240.

Substitutions in four different positions spanning residues 227 to 240 were found to produce a sigma-specific effect. The S233 position was identified twice in our screen. The change in each case was from serine to proline. In the work of Buckner et al. (8), a mutant in which serine was replaced by an alanine rather than proline at this position was found not to exhibit any defect. This suggests that the S233 residue is not directly involved in transcriptional activation; most likely, the S233P substitution causes a local conformational change in the peptide backbone that repositions other residues that make critical contacts with σ. However, the effect is unlikely to be profoundly disruptive, as the level of *spoIIA* expression for this mutant was nearly the same as the wild-type level. The most likely candidate for direct involvement in transcription activation and perhaps an interaction with the transcription machinery is the valine at position 240. Conservative changes at this residue cause a drastic decrease in the expression of the σ^A-dependent promoters while still maintaining expression at the σ^H-dependent promoters. One point that we did not address directly in this study was the contribution of the K265 residue to the defect seen in the V240G K265R mutant. However, the isolation of a second change at position 240, a valine-to-alanine single substitution, implies that the residue at position 240 is important.

Despite our success in identifying a region that is implicated in activation of one class of Spo0A-dependent promoters, we cannot rule out the possibility that other residues may be involved in transcription activation at the σ^A-dependent promoters. The screen, although remarkable in identifying a narrow region of the Spo0A protein as important for activation of σ^A-dependent promoters, was not a saturating screen. Moreover, C-terminal mutations were more likely to be isolated by integration of a vector carrying promoterless copies of mutant *spo0A* genes into the *B. subtilis* chromosome. We are confident, however, that our screen did include representatives of mutations in the N-terminal end of the protein, because some of the mutants we isolated also contained additional silent mutations in the N-terminal ends of their coding sequences (data not shown). Subtle changes in Spo0A function were most likely overlooked by our screen, since we focused on those strains that exhibited the strongest phenotypes in our primary screen. In addition, our screen failed to identify mutants of Spo0A specifically defective at σ^H-dependent promoters. This result might be due to the complex regulatory relationship between Spo0A and σ^H because of the roles they play in each other's expression (36). Therefore, it is possible that mutations that affect Spo0A function at σ^H-dependent promoters affect levels of Spo0A itself and perhaps result in a null phenotype. However, the existence of the *spo0A9V* mutant indicates that it is possible to obtain Spo0A mutants defective in stimulating transcription at σ^H-dependent promoters that still maintain the ability to bind DNA (28).

The existence of mutant forms of Spo0A that are specifically defective in their ability to activate transcription from promoters utilized by σ^A-associated holoenzyme is consistent with two

mechanistic models. One possibility is that Spo0A binds differently to promoters recognized by the two forms of RNAP holoenzyme. Although this possibility cannot be excluded, and 0A boxes most critical for transcription activation actually overlap polymerase-binding sites, affinity for 0A boxes depends upon a consensus sequence that is independent of promoter context. We therefore favor the alternative explanation that mutations producing a sigma-specific phenotype do so by disrupting a sigma-specific contact between Spo0A and RNAP. The results of Buckner et al. (8) are also most easily explained by such a model. Nevertheless, we note the suggestion by Bird et al. (6) that the target for phosphorylated Spo0A binding is not DNA per se but rather DNA plus RNAP, and we acknowledge the possibility that holoenzyme-specific defects might reflect a more complex combination of DNA and protein interactions.

In this study, we have identified an activating region of Spo0A specific for σ^A-dependent transcription which may interact directly with the sigma subunit of RNAP to activate transcription. The isolation of a region specific for interaction with σ^A and promotion of σ^A-dependent transcription suggests that a separate surface of Spo0A may exist for interaction with the σ^H factor at σ^H-dependent promoters. The ability of transcriptional activators and components of RNAP to interact in many different ways and on many different surfaces could greatly increase the available combinations of potential regulatory interactions between these proteins. The elucidation of the mechanism of transcriptional activation by Spo0A still waits on the identification of the DNA binding domain and residues important for activation at σ^H-dependent promoters.

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