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Essential Internal Promoter in the *spoIIIA* Locus of *Bacillus subtilis*[▽]

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The *Bacillus subtilis spoIIIA* locus encodes eight proteins, SpoIIIAA to SpoIIIAH, which are expressed in the mother cell during endospore formation and which are essential for the activation of σ^G in the forespore. Complementation studies indicated that this locus may be transcribed from two promoters, one promoter upstream from the first gene and possibly a second unidentified promoter within the locus. Fragments of the *spoIIIA* locus were expressed at an ectopic site to complement the sporulation-defective phenotype of a *spoIIIAH* deletion, and we determined that complementation required a fragment of DNA that extended into *spoIIIAF*. To confirm that there was a promoter located in *spoIIIAF*, we constructed transcriptional fusions to *lacZ* and found strong sporulation-induced promoter activity. Primer extension assays were used to determine the transcription start site, and point mutations introduced into the −10 and −35 regions of the promoter reduced its activity. This promoter is transcribed by σ^E-RNA polymerase and is repressed by SpoIIID. Therefore, we concluded that the *spoIIIA* locus is transcribed from two promoters, one at the start of the locus ($P1_{spoIIIA}$) and the other within the locus ($P2_{spoIIIA}$). Based on Campbell integrations and reverse transcription-PCR analysis of the $P2_{spoIIIA}$ region, we determined that $P2_{spoIIIA}$ is sufficient for transcription of *spoIIIAG* and *spoIIIAH*. Inactivation of $P2_{spoIIIA}$ blocked spore formation, indicating that $P2_{spoIIIA}$ is essential for expression of *spoIIIAG* and *spoIIIAH*. The $P2_{spoIIIA}$ activity is twice the $P1_{spoIIIA}$ activity; therefore, larger amounts of SpoIIIAG and SpoIIIAH than of proteins encoded at the upstream end of the locus may be required.

In response to nutrient depletion, *Bacillus subtilis* can respond by differentiating into an endospore, which can remain dormant until nutrients become available to stimulate its germination. Soon after the onset of endospore formation, the cell divides into two morphologically distinct cells, the larger mother cell and the smaller forespore. During sporulation, 383 genes are expressed in the mother cell (8), while 143 different genes are expressed in the forespore (23). The cell type-specific patterns, as well as the temporal patterns of gene expression, are governed largely by the appearance and activation of four RNA polymerase sigma factors (for reviews, see references 9, 12, and 17). The activity of each of these sigma factors is tightly regulated for two purposes, thereby coupling the activation of gene expression to the completion of morphological landmarks and synchronizing the developmental programs of gene expression in each of the two cell types. For example, activation of σ^G in the forespore requires σ^E-directed gene expression in the mother cell.

Transcription of the *spoIIIA* locus by σ^E is required for σ^G activation (13, 15). The *spoIIIA* locus, which is conserved throughout spore-forming bacteria, encodes eight proteins, SpoIIIAA to SpoIIIAH, each of which is required for σ^G activation (15). Seven of the eight products, SpoIIIAB to SpoIIIAH, are predicted to be membrane associated (4, 20, 22); SpoIIIAA shows homology to AAA-type ATPases, but the other seven proteins show homology only to their orthologs in other sporulating bacteria. SpoIIIAH interacts with the forespore-expressed protein SpoIIQ via the large extracellular C-terminal domains (1, 6, 18). The two proteins make contact first at the septum between the mother cell and the forespore and then migrate with the mother cell membrane as the forespore is engulfed (1, 2). It is not known how this complex contributes to σ^G activation or what roles the other *spoIIIA*-encoded proteins play.

Transcription of the *spoIIIA* locus is initiated at a σ^E-dependent promoter located immediately upstream of *spoIIIAA* (13). Transcription from this promoter is repressed by the DNA binding protein SpoIIID (13) and possibly to a lesser extent by GerR (8). Transcription from the gene immediately preceding the *spoIIIA* locus, *yqhV*, may also read into the *spoIIIA* locus since there is no obvious transcription terminator between the two genes. Since *yqhV* transcription is also σ^E dependent and repressed by GerR, this may account for the effect of GerR on *spoIIIA* transcription (8).

A number of operons found in both gram-positive and gram-negative bacteria are transcribed from more than one promoter. In some instances, these promoters are expressed under different conditions, and their activities are controlled by different RNA polymerase sigma factors or DNA binding proteins (3, 21). The use of alternative promoters can also allow different levels of expression of different genes in the locus in response to different signals (16, 24).

Two observations led us to suspect that a second promoter located within the *spoIIIA* locus may play a role in its expression. A transposon insertion into the extreme 5′ end of *spoIIIAA* does not prevent expression of SpoIIIAH, as determined by Western blotting (1). We also noted that when SpoIIIAH was directly expressed from the promoter located upstream from *spoIIIAA* ($P1_{spoIIIA}$) at an ectopic locus, this construct did not complement the sporulation defect of a *spoIIIAH* mutant strain. Moreover, a second putative promoter within the locus had been proposed previously (19a). Therefore, we began a search for the promoter responsible for *spoIIIAH* transcription.

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TABLE 1. Plasmids used in this study

Plasmid	Genotype	Reference or source
pDG1661		10
pDG1662		10
pDG784		9
pDG1515		9
pDG1726		9
pMS38		Lab stock
pLitmus28		NEB
pCG123	pDG1662 + *spoIIIAH*	This study
pCG124	pDG1662 + *spoIIIAGH*	This study
pCG125	pDG1662 + *spoIIIAFGH*	This study
pCG126	pDG1662 + *spoIIIA′EFGH* (bp 1010 to 1215 of *spoIIIAE*)	This study
pCG129	pDG1661 + *spoIIIAH*	This study
pCG130	pDG1661 + *spoIIIAGH*	This study
pCG131	pDG1661 + *spoIIIAFGH*	This study
pCG132	pDG1661 + *spoIIIA′EFGH*	This study
pCG134	pDG1661 + $P1_{spoIIIA}$	This study
pCG135	pDG1661 + *spoIIIAFGH* (bp 1 to 42 of *spoIIIAH*)	This study
pCG136	pDG1661 + *spoIIIAFG* (bp 1 to 42 of *spoIIIAG*)	This study
pCG137	pDG1661 + *spoIIIAF*	This study
pCG138	pDG1661 + *spoIIIAF* (200 bp 3′ of *spoIIIAF* deleted)	This study
pCG139	pDG1661 + *spoIIIAF* (400 bp 3′ of *spoIIIAF* deleted)	This study
pCG140	pDG1661 + *spoIIIA′EFGH* (bp 1 to 42 *spoIIIAH*)	This study
pCG141	pDG1661 + *spoIIIA′EFG* (bp 1 to 42 *spoIIIAG*)	This study
pCG142	pDG1661 + *spoIIIA′EF*	This study
pCG143	pDG1661 + *spoIIIA′EF* (200 bp 3′ of *spoIIIAF* deleted)[a]	This study
pCG144	pDG1661 + *spoIIIA′EF* (400 bp 3′ of *spoIIIAF* deleted)	This study
pCG145	pDG1661 + *spoIIIA′EF* (600 bp 3′ of *spoIIIAF* deleted)	This study
pCG146	pDG1661 + *spoIIIA′E*	This study
pCG149	pDG1661 + *spoIIIAF-lacZ* (100 bp 5′ of *spoIIIAF* deleted)	This study
pCG150	pDG1661 + *spoIIIAF-lacZ* (200 bp 5′ of *spoIIIAF* deleted)[b]	This study
pCG151	pDG1661 + *spoIIIAF-lacZ* (300 bp 5′ of *spoIIIAF* deleted)	This study
pCG152	pMS38 + *spoIIIAF*	This study
pCG153	pMS38 + *spoIIIAE*	This study
pCG154	pMS38 + *spoIIIAD*	This study
pCG155	pMS38 + *spoIIIAC*	This study
pCG156	pMS38 + *spoIIIAB*	This study
pCG162	pDG1726 Δ*gerR*	This study
pCG163	pDG784 Δ*spoIIID*	This study
pCG164	pDG1726 Δ*sigE*	This study
pCG165	pDG1515 Δ*sigK*	This study
pCG166	pLitmus28 + *spoIIIAFGH*	This study
pCG167	pDG1661 + P_{sspE} (bp −165 to 14)	This study
pCG169	pLitmus28 + *spoIIIAFGH* −30 C-to-T mutation	This study
pCG170	pLitmus28 + *spoIIIAFGH* −12/−11 CA-to-GT mutation	This study
pCG179	pDG1661 + $P2_{spoIIIA}$ −30 C-to-T mutation	This study
pCG180	pDG1661 + $P2_{spoIIIA}$ −12/−11 CA-to-GT mutation	This study
pCG184	pDG1661 + *spoIIIAFGH* −12/−11 CA-to-GT mutation	This study
pCG185	pDG1662 + *spoIIIAFGH* −12/−11 CA-to-GT mutation	This study
pCG202	pDG784 + *spoIIIAFGH* −12/−11 CA-to-GT mutation	This study

[a] This site was used as 3′ end for all remaining experiments.
[b] The fragment of DNA from base 200 to base 415 of *spoIIIAF* is referred to as $P2_{spoIIIA}$.

We found a promoter ($P2_{spoIIIA}$) that is located in *spoIIIAF* and is necessary and sufficient for expression of *spoIIIAG* and *spoIIIAH*.

MATERIALS AND METHODS

Bacterial strains and culture media. The strains and plasmids used in this study are shown in Tables 1 and 2. Routine microbiological manipulations and procedures were carried out as described by Cutting and Harwood (5). The

TABLE 2. *B. subtilis* strains used in this study

Strain	Genotype	Source or reference
JH642	*trpC2 pheA1*	J. Hoch
AOB114	MB24 *gerE::kan*	Lab stock
RL2045	Δ*spoIIIAG*	P. Stragier
RL2046	Δ*spoIIIAH*	P. Stragier
CGB125	*amyE::spoIIIAH*	This study
CGB126	*amyE::spoIIIAGH*	This study
CGB127	*amyE::spoIIIAFGH*	This study
CGB128	*amyE::spoIII′AEFGH*	This study
CGB129	RL2046 *amyE::spoIIIAH*	This study
CGB130	RL2046 *amyE::spoIIIAGH*	This study
CGB131	RL2046 *amyE::spoIIIAFGH*	This study
CGB132	RL2046 *amyE::spoIII′AEFGH*	This study
CGB133	RL2045 *amyE::spoIIIAH*	This study
CGB134	RL2045 *amyE::spoIIIAGH*	This study
CGB135	RL2045 *amyE::spoIIIAFGH*	This study
CGB136	RL2045 *amyE::spoIIIA′EFGH*	This study
CGB142	*amyE::spoIIIAH-lacZ*	This study
CGB143	*amyE::spoIIIAGH-lacZ*	This study
CGB144	*amyE::spoIIIAFGH-lacZ*	This study
CGB145	*amyE::spoIIIA′EFGH-lacZ* (last 200 bp of *spoIIIAE*)	This study
CGB147	*amyE::*$P1_{spoIIIA}$*-lacZ*	This study
CGB158	*amyE::spoIIIAFGH-lacZ* (42 bp of *spoIIIAH*)	This study
CGB159	*amyE::spoIIIAFG-lacZ* (42 bp of *spoIIIAG*)	This study
CGB160	*amyE::spoIIIAF-lacZ*	This study
CGB161	*amyE::spoIIIAF-lacZ* (200 bp at 3′ end of *spoIIIAF* deleted)	This study
CGB162	*amyE::spoIIIAF-lacZ* (400 bp at 3′ end of *spoIIIAF* deleted)	This study
CGB163	*amyE::spoIIIA′EFGH-lacZ* (42 bp of *spoIIIAH*)	This study
CGB164	*amyE::spoIIIA′EFG-lacZ* (42 bp of *spoIIIAG*)	This study
CGB165	*amyE::spoIIIA′EF-lacZ* (200 bp at 3′ end of *spoIIIAF* deleted)[a]	This study
CGB166	*amyE::spoIIIA′EF-lacZ* (400 bp at 3′ end of *spoIIIAF* deleted)	This study
CGB167	*amyE::spoIIIA′EF-lacZ* (600 bp at 3′ end of *spoIIIAF* deleted)	This study
CGB168	*amyE::spoIIIA′E-lacZ* (last 200 bp of *spoIIIAE*)	This study
CGB172	*amyE::spoIIIAF-lacZ* (100 bp at 5′ end of *spoIIIAF* deleted)	This study
CGB173	*amyE::spoIIIAF-lacZ* (200 bp at 5′ end of *spoIIIAF* deleted)[b]	This study
CGB174	*amyE::spoIIIAF-lacZ* (300 bp at 5′ end of *spoIIIAF* deleted)	This study
CGB175	*spoIIIAF* Campbell	This study
CGB176	*spoIIIAE* Campbell	This study
CGB177	*spoIIIAD* Campbell	This study
CGB178	*spoIIIAC* Campbell	This study
CGB179	*spoIIIAB* Campbell	This study
CGB187	*gerR::spec amyE::*$P1_{spoIIIA}$	This study
CGB188	*spoIIID::kan amyE::*$P1_{spoIIIA}$	This study
CGB189	*sigE::spec amyE::*$P1_{spoIIIA}$	This study
CGB190	*sigK::tet amyE::*$P1_{spoIIIA}$	This study
CGB191	*gerE::kan amyE::*$P1_{spoIIIA}$	This study
CGB192	*gerR::spec amyE::*$P2_{spoIIIA}$	This study
CGB193	*spoIIID::kan amyE::*$P2_{spoIIIA}$	This study
CGB194	*sigE::spec amyE::*$P2_{spoIIIA}$	This study
CGB195	*sigK::tet amyE::*$P2_{spoIIIA}$	This study
CGB196	*gerE::kan amyE::*$P2_{spoIIIA}$	This study
CGB197	*amyE::*P_{sspE}*-lacZ*	This study
CGB198	CGB175 *amyE::*P_{sspE}*-lacZ*	This study
CGB202	*amyE::*$P2_{spoIIIA}$*-lacZ* (−30 C-to-T mutation)	This study
CGB203	*amyE::*$P2_{spoIIIA}$*-lacZ* (−12/−11 CA-to-GT mutation)	This study
CGB206	*amyE::spoIIIAFGH-lacZ* (−12/−11 CA-to-GT mutation)	This study
CGB207	*amyE::spoIIIAFGH* (−12/−11 CA-to-GT mutation)	This study
CGB208	RL2045 *amyE::spoIIIAFGH* (−12/−11 CA-to-GT mutation)	This study
CGB209	RL2046 *amyE::spoIIIAFGH* (−12/−11 CA-to-GT mutation)	This study
CGB225	*spoIIIAFGH* (−12/−11 CA-to-GT mutation)	This study
CGB226	CGB197 *spoIIIAFGH* (−12/−11 CA-to-GT mutation)	This study
CGB227	CGB225 *amyE::spoIIIAFGH-lacZ*	This study

[a] This site was used as the 3′ end for all remaining experiments.
[b] The fragment of DNA from base 200 to base 415 of *spoIIIAF* is referred to as $P2_{spoIIIA}$.

concentrations of antibiotics used for selection in Luria broth (LB) and Difco sporulation medium (DSM) were as follows: 100 μg/ml ampicillin, 5 μg/ml chloramphenicol, 10 μg/ml kanamycin, 100 μg/ml spectinomycin, and 20 μg/ml tetracycline. Cultures were grown in LB, and sporulation was induced by nutrient exhaustion in DSM. Competent cells were prepared and transformed using the Spizizen method (5).

To construct the plasmids used for complementation of the sporulation-defective phenotype of RL2045 (JH642 Δ*spoIIIAG*) and RL2046 (JH642 Δ*spoIIIAH*), fragments of DNA corresponding to *spoIIIAH*, *spoIIIAGH*, *spoIIIAFGH* and *spoIIIA′EFGH* were amplified from JH642 chromosomal DNA, cloned into pDG1662 (11) digested with BamHI and EcoRI, and transformed into competent JH642, RL2045, and RL2046 with selection for chloramphenicol resistance and screening for spectinomycin sensitivity to confirm double crossovers into the *amyE* locus.

In order to more positively identify which region of DNA contained the putative promoter, we amplified the sections of DNA described above and cloned them into EcoRI- and BamHI-digested pDG1661 (11), which contains a promoterless *lacZ* gene fused to the ribosome binding site of *spoVG*, and then we transformed the resulting plasmids into competent JH642, with selection for chloramphenicol resistance and screening for spectinomycin sensitivity to confirm double crossovers into the *amyE* locus. To compare the known *spoIIIA* operon promoter $P1_{spoIIIA}$ (upstream of *spoIIIAA*) to $P2_{spoIIIA}$, we amplified DNA from position −246 to position 7 relative to the $P1_{spoIIIA}$ transcription start site and cloned it into pDG1661 as described above.

Since we wanted to define the $P2_{spoIIIA}$ promoter region more narrowly, we made sequential deletions from the 5′ and 3′ ends of the *spoIIIAF* gene. First, we kept the 5′ end constant either at the second codon of *spoIIIAF* or at base 1016 of *spoIIIAE*, and the 3′ end was moved inward from the last codon of *spoIIIAF* to the second codon of *spoIIIAF* in 200-bp intervals. DNA fragments resulting from the PCR amplifications were cloned into pDG1661 digested with EcoRI and BamHI and transformed into competent JH642 as described above. Once a suitable 3′ end was established, as determined by β-galactosidase activity, we constructed and transformed pDG1661-based plasmids as described above that had DNA fragments with successive 100-bp deletions from the second codon of *spoIIIAF*.

The following double-crossover knockouts of potential $P2_{spoIIIA}$ regulators were constructed in early and late stages of mother cell transcription: (i) σ^E and regulators SpoIIID and GerR and (ii) σ^K and a regulator GerE. Approximately 500 bp of 5′- and 3′-flanking DNA of each gene was amplified from chromosomal DNA and cloned into vectors designed for gene replacement. First *gerR* and *sigE* 5′ homology regions were cloned into EcoRV- and EcoRI-digested pDG1726 (10) (spectinomycin), and then the 3′ homology regions were cloned into the resulting plasmids with BamHI and SalI. The *spoIIID* 5′ homology region was cloned into EcoRI- and BamHI-digested pDG784 (10) (kanamycin), and the 3′ region was cloned into the resulting plasmid with PstI and SphI. Homology regions for *sigK* were cloned first into the EagI and BamHI sites of pDG1515 (10) (tetracycline) and then into the EcoRI and HindIII sites of the resulting plasmid. Each of the final plasmids was linearized with PstI or ScaI and transformed into competent CGB147 and CGB173 with selection for the appropriate antibiotic marker. Double crossovers were confirmed by PCR analysis. The *gerE* knockout was constructed by transforming competent CGB147 and CGB173 with chromosomal DNA isolated from laboratory stock strain AOB114 (MB24 *gerE*::*kan*) and selecting for kanamycin resistance.

To introduce point mutations into the −35 and −10 regions of $P2_{spoIIIA}$ and into both potential alternative start codons for SpoIIIAG, we designed two complementary QuikChange oligonucleotides per desired mutation and performed QuikChange mutagenic PCR on pCG166 as described below. After sequencing to ensure the presence of the desired mutation, we subcloned (i) the previously defined promoter region into pDG1661 containing the separate −30 and −12/−11 mutations using EcoRI and BamHI and (ii) the entire *spoIIIAFGH* fragment containing the −12/−11 point mutations into both pDG1661 and pDG1662 using BamHI and EcoRI. The four resulting plasmids were transformed into competent JH642 (or RL2045 and RL2046 for pDG1662 derivatives) with selection for chloramphenicol resistance and screening for spectinomycin sensitivity to confirm double crossovers into the *amyE* gene.

Plasmids for Campbell integration into *spoIIIAF*, *spoIIIAE*, *spoIIIAD*, *spoIIIAC*, and *spoIIIAB* were constructed by amplifying each entire gene from chromosomal DNA along with its ribosome binding site and a few bases downstream of the stop codon and cloning the DNA into pMS38 digested with EcoRV and BamHI. The resulting plasmids were transformed into competent JH642 with selection for chloramphenicol resistance. The presence of single crossovers was confirmed by amplification of the ampicillin resistance gene on the pMS38 vector backbone from chromosomal DNA.

The promoter region of *sspE*, beginning 130 bp upstream of the −35 region and extending 5 bases past its ribosome binding site, was amplified from chromosomal DNA and cloned into pDG1661 digested with BamHI and HindIII. The plasmid was transformed into competent JH642 and CGB175 with selection for chloramphenicol resistance and screening for spectinomycin sensitivity to confirm double crossovers into the *amyE* gene.

In order to isolate a strain with the two base pair substitutions in the −10 region of $P2_{spoIIIA}$ at the *spoIIIA* chromosomal locus, we cloned the *spoIIIAFGH* DNA fragment harboring the −12/−11 CA-to-GT $P2_{spoIIIA}$ mutation from pCG170 into pDG784 (10) that had been cleaved with EcoRI and BamHI. In the second step, an approximately 500-bp fragment of DNA from the region immediately downstream of *spoIIIAH* was amplified and cloned into the pDG784 derivative between the PstI and SphI sites. The resulting plasmid was used to transform competent JH642 and CGB197 to kanamycin resistance. PCR amplification and DNA sequencing of the transformants were used to show that a double crossover resulted in replacement of the wild-type copy of $P2_{spoIIIA}$ with the mutated $P2_{spoIIIA}$, resulting in strains CGB225 and CGB226, respectively. To test for complementation of the sporulation defect of CGB225, we isolated a derivative of CGB225 in which *spoIIIAFGH* was inserted at *amyE*.

Sporulation efficiency test. In order to determine the sporulation efficiency of *B. subtilis* strains, 5-ml cultures were incubated in DSM (supplemented with appropriate antibiotics for Campbell insertion strains) for approximately 48 h at 37°C (for 24 h at 37°C for point mutants). Aliquots of each culture were heated at 80°C for 10 min, serially diluted, and plated on LB agar (with appropriate antibiotics for Campbell strains). Unheated aliquots were also serially diluted and plated. Colonies were counted after 16 h of incubation at 37°C.

β-Galactosidase assays. Overnight LB cultures (incubated at 30°C for point mutants and at 37°C for other strains) were diluted 1:100 in fresh DSM with 5 μg/ml chloramphenicol (100 μg/ml spectinomycin for CGB197 and its derivatives) and allowed to grow for 2 h at 37°C. Two 300-μl aliquots were collected every 0.5 h until 6 h after the onset of sporulation (usually 8 to 8.5 h); the first aliquot was used to measure the optical density at 600 nm, while the second aliquot was spun down and the cell pellet was stored at −80°C until it was assayed for β-galactosidase activity (5).

RNA isolation. RNA was isolated from sporulating *B. subtilis* strains 4 h after the onset of sporulation using the Epicenter MasterPure RNA purification kit protocol (catalog no. MCR85102; Epicenter Biotechnologies), with the following modifications: 2 ml of RNAprotect bacterial reagent (catalog no. 76506; QIAGEN) was added to 1 ml of a culture and incubated 5 min at room temperature before cells were harvested. Six hundred microliters of tissue and cell lysis solution was added to the cell pellets, which were resuspended by vortexing. The cell suspension was placed in a tube containing lysing matrix B (catalog no. 6911-100; MP Biomedical) and then lysed in a Bio101 Fastprep FP120 machine (MP Biomedical) three times at speed 6 for 45 s. The glass beads were spun down by centrifugation at 16,100 × *g* for 2 min, and 300 μl of supernatant was transferred to a clean tube. Proteinase K was added as described in the kit protocol instructions, and the protocol was followed until the DNase I digestion step (step C). At this point, we incubated samples for 30 min after DNase I addition and repeated the entire step C to ensure that all DNA was removed. RNA was quantitated by spectrophotometric analysis at 260 nm.

Primer extension. Thirty picomoles of oligonucleotide primer *lacZ*-rev2 (Table 3) (100 pmol/μl) was end labeled with [γ-^{32}P]ATP (Amersham Biosciences) according to the protocol for an Epicenter SequiTherm EXCEL II DNA sequencing kit (catalog no. SEM79020; Epicenter Biotechnologies), except that we incubated the reaction mixture for 60 min at 37°C and terminated the reaction by incubation at 70°C for 10 min. Labeled primer was purified over a MicroSpin G25 column (Amersham Biosciences) as described in the product manual. Six picomoles of purified labeled primer was added to 1.7 μg of total RNA from CGB173, CGB193, and CGB194. cDNA synthesis was performed according to the Invitrogen ThermoScript reverse transcription (RT)-PCR system (catalog no. 11146-024; Invitrogen) protocol using a cDNA synthesis temperature of 55°C.

For sequencing, 1.5 pmol of purified labeled primer was used along with 50 fmol of pCG150 according to the Epicenter SequiTherm EXCEL II DNA sequencing kit (catalog no. SEM79020; Epicenter Biotechnologies) protocol, except that during cycle sequencing we added a 30-s annealing step at 55°C. Before electrophoresis on a 6% polyacrylamide gel with 6 M urea, loading buffer was added to all samples and the samples were heated at 90°C for 10 min. Samples were electrophoresed in 1× Tris-borate-EDTA, (pH 8.3) at 65 W for 2 to 2.5 h or until the xylene cyanol had just run off the gel. The gel was dried and exposed overnight to an Amersham Biosciences phosphor screen.

Site-directed mutagenesis. Point mutations were introduced as described in the manual for a Stratagene QuikChange site-directed mutagenesis kit (catalog

TABLE 3. Oligonucleotide primers used for PCR, sequencing, and mutagenesis[a]

Primer	Sequence (5′ to 3′)
spoIIIAH-rev-EcoRI	CACACACA**GAATTC**TTTAGCGGGCTTTTTTCCCTCATTCTTA
spoIIIAH-for-BamHI	CACACACA**GGATCC**CTTAAAAAACAAACCGTTTGGCTATT
spoIIIAG-for-BamHI	CACACACA**GGATCC**AATAAAAACGGATTATGGAATGTA
spoIIIAF-for-BamHI	CACACACA**GGATCC**AGTTTTTTAACGGAATGGCTTACC
spoIIIAE-for-BamHI	CACACACA**GGATCC**GTAATCAGCGCCTCTTTACT
spoIIIAH-rev-BamHI	CACACACA**GGATCC**TTTAGCGGGCTTTTTTCCCTCATTC
spoIIIAH-for-EcoRI	CACACACA**GAATTC**CTTAAAAAACAAACCGTTTGGCTATT
spoIIIAG-for-EcoRI	CACACACA**GAATTC**AATAAAAACGGATTATGGAATGTA
spoIIIAF-for-EcoRI	CACACACA**GAATTC**AGTTTTTTAACGGAATGGCTTACC
spoIIIAE-for-EcoRI	CACACACA**GAATTC**GTAATCAGCGCCTCTTTACT
spoIIIAH-rev-fuse-BamHI	CACACACA**GGATCC**TAAGACTGAGCATTGTTAATAGCCAAA
spoIIIAG-rev-fuse-BamHI	CACACACA**GGATCC**TAAGAGACTGCTTTTTCAATACATTCCA
spoIIIAF-rev-*Bam*HIA	CACACACA**GGATCC**TTTCATTGCCGACACTCTC
spoIIIAF-rev-*Bam*HIB	CACACACA**GGATCC**TGGCCATATACACGCTGATTGTTTT
spoIIIAF-rev-*Bam*HIC	CACACACACA**GGATCC**TCTCTGACTGCCCGTTTTT
spoIIIAE-rev-BamHI	CACACACA**GGATCC**ATGAGAGACACAATGGCGAGA
spoIIIAF-for-*Eco*RIA	CACACACA**GAATTC**ATGGTAGTCAGCCTGCTCTTGATT
spoIIIAF-for-*Eco*RIB	CACACACA**GAATTC**AAACGGGCAGTCAGAGTCT
spoIIIAF-for-*Eco*RIC	CACACACA**GAATTC**AATGGCTGTCCAACTAAA
lacZ-rev2	ATCTTACGTCAGTAACTTCCACAGT
P*spoIIIA*-for-EcoRI	CACACACA**GAATTC**ACGGCAGCAATTGTCATGCTTGTGA
P*spoIIIA*-rev-BamHI	CACACACA**GGATCC**ATTGGCTTCTTTAAAATGTATGATGTGAG
spoIIIAF-for-*Eco*RV	CACACACA**GATATC**TCACGATGATGATGAAATGAAGGA
spoIIIAF-rev-BamHI	CACACACA**GGATCC**GAAGAGACTGCTTTTTCAATACAT
spoIIIAE-for-*Eco*RV	CACACACA**GATATC**TGTCATAACCGAAAGGAGGCGGTA
spoIIIAE-rev-BamHI	CACACACA**GGATCC**ACAACACGAATGGTGGTAAGC
spoIIIAD-for-*Eco*RV	CACACACA**GATATC**AGCTGTGTTTTTATTCCAAGGATAGG
spoIIIAD-rev-BamHI	CACACACA**GGATCC**TTCAATCTACCGCCTCCTTT
spoIIIAC-for-*Eco*RV	CACACACA**GATATC**GATGTAAACGTGAGGGGAGCAA
spoIIIAC-rev-BamHI	CACACACA**GGATCC**AATGTCAATCTGCAAGCCCCCCTAT
spoIIIAB-for-*Eco*RV	CACACACA**GATATC**AAATTTATGACAAAGACGGAAATGTG
spoIIIAB-rev-BamHI	CACACACA**GGATCC**TTTATTTTTGCTCCCCTCACGTTA
gerR-for-del-*Eco*RV	CACACACA**GATATC**CTGAACAAGAAAAAGGAGCTGCTC
gerR-rev-del-EcoRI	CACACACA**GAATTC**AGTCCAAGCATCTTGTCTTGTAATGGT
gerR-for-del-BamHI	CACACACA**GGATCC**ATGAAAAAAGCGGCTCAAGAA
gerR-rev-del-SalI	CACACACA**GTCGAC**TCAAAGAATACGGCATTCAGGA
spoIIID-for-del-EcoRI	CACACACA**GAATTC**GAGTCATTGGTCCGATCGTA
spoIIID-rev-del-BamHI	CACACACA**GGATCC**TGTTCGCTCTTTGATGTAATCGTGCA
spoIIID-for-del-PstI	CACACACA**CTGCAG**GAAGGAGAGCCTGTTCAGCAAT
spoIIID-rev-del-SphI	CACACACA**GCATGC**TTTGAGAACAGGCCTTTTACA
sigE-for-del-*Eco*RV	CACACACA**GATATC**GTCAGCGTGCAGGCAGATT
sigE-rev-del-EcoRI	CACACACA**GAATTCC**AGCAGCTTATACCAGAGGTGCGTCAA
sigE-for-del-BamHI	CACACACA**GGATCC**AAAGAGTTCAACAAAATGGTG
sigE-rev-del-SalI	CACACACA**GTCGAC**TTGTAAGCGATGTCCC
sigK-for-del-EagI	CACACACA**CGGCCG**ACAATAAACAGCACTCTGGTACC
sigK-rev-del-BamHI	CACACACA**GGATCC**AACAAAGCCGAGCGCTGCGAAAA
sigK-for-del-EcoRI	CACACACA**GAATTC**ATCTTCAAGAGTTAAGTTATCGCACCGAT
sigK-rev-del-HindIII	CACACACA**AAGCTT**GGTTTGGTTAGATGCGGAAAATGC
AF35for	CAAAAAAAATAGAAA<u>C</u>ACAAGCTTCCCAGCG
AF35rev	CGCTGGGAAGCTTGT<u>G</u>TTTCTATTTTTTTTG
AF10for	CAAGCTTCCCAGCGCG<u>GT</u>TATATTCTAGAAGAAATGG
AF10rev	CCATTTCTTCTAGAATATA<u>AC</u>CGCGCTGGGAAGCTTG
sspE-for-HindIII	CACACACA**AAGCTT**ACGCATGGTCGAAATTAAAGAC
sspE-rev-BamHI	CACACACA**GGATCC**TGTTATCACCTCCACGGTCA

[a] Restriction enzyme sites are indicated by bold type, and base changes in QuikChange primers are indicated by underlining.

no. 200518; Stratagene) with the following PCR cycle parameters: one cycle of 95°C for 1 min, followed by 18 cycles of 95°C for 30 s, 55°C for 1 min, and 68°C for 7 min and a final extension step of 10 min at 68°C. One microliter of DpnI was added to each reaction mixture, and the tubes were incubated at 37°C for 1 to 2 h. Five to seven microliters of each reaction mixture was then transformed into One Shot Top10 chemically competent cells (Invitrogen) as described in the product manual. The entire transformation reaction mixture was plated on LB plates containing 100 μg/ml ampicillin. Individual colonies were grown in liquid culture, and plasmid DNA was isolated (QIAGEN QIAprep spin miniprep kit) and sequenced to ensure that the desired mutation was present.

RT-PCR. RNA was prepared from wild-type *B. subtilis* JH642 at 4 h after the onset of sporulation as described above and used to make cDNA as described above, except that primer *spoIIIAF*-rev-*Bam*HIB was used. PCR was then performed using primers *spoIIIAF*-for-*Eco*RIA and *spoIIIAF*-for-*Eco*RIC with *spoIIIAF*-rev-*Bam*HIB and Platinum *Taq* high-fidelity DNA polymerase (Invitrogen) for 30 cycles according to the ThermoScript RT-PCR system protocol (Invitrogen). PCR products were electrophoresed on a 1.5% agarose gel. A negative control containing no reverse transcriptase was included for each primer set.

RESULTS

Second promoter within the *spoIIIA* locus. To search for the promoter that drives expression of *spoIIIAG* and *spoIIIAH*

TABLE 4. Complementation of Δ*spoIIIAG* and Δ*spoIIIAH* by *spoIIIA* fragments

DNA fragment at *amyE*	Δ*spoIIIAG*[a]		Δ*spoIIIAH*[b]	
	No. of heat-resistant spores formed/ml	Complementation (% relative to wild type)	No. of heat-resistant spores formed/ml	Complementation (% relative to wild type)
spoIIIAH	1.1×10^4	0.02	2.2×10^4	0.03
spoIIIAGH	2.9×10^4	0.05	3.9×10^3	0.002
spoIIIAFGH	1.3×10^8	99	1.1×10^8	97
spoIIIA′EFGH	1.25×10^8	98	1.04×10^8	96
spoIIIAFGH −12/−11[c]	4.5×10^4	0.07	1.7×10^4	0.025
$P1_{spoIIIA}$-*spoIIIAH*[d]	ND[e]	ND	6.3×10^4	0.09

[a] *spoIIIA* fragments in RL2045 (Δ*spoIIIAG*) and strains CGB129, CGB130, CGB131, and CGB32.
[b] *spoIIIA* fragments in RL2046 (Δ*spoIIIAH*) and strains CGB133, CGB134, CGB135, and CGB136.
[c] *spoIIIAFGH* fragment with base substitutions at positions −12 and −11 of $P2_{spoIIIA}$ (strains CGB208 and CGB209).
[d] Fusion in only RL2046. *spoIIIAH* was directly expressed from $P1_{spoIIIA}$ at *amyE*.
[e] ND, not determined.

independent of the upstream *spoIIIA* genes, we tested the ability of fragments of the *spoIIIA* operon inserted into the chromosome at *amyE* to complement the sporulation-defective phenotypes of RL2045 and RL2046, which have in-frame nonpolar deletions of *spoIIIAG* and *spoIIIAH*, respectively. Only the fragments of DNA which contained DNA extending from *spoIIIAF* through *spoIIIAH* were able to complement mutations in *spoIIIAG* or *spoIIIAH* (Table 4). Since a region in *spoIIIAF* was required for complementation of *spoIIIAH* and *spoIIIAG* mutants, we hypothesized that a promoter is located within *spoIIIAF*. To test this hypothesis, the same DNA fragments of the *spoIIIA* locus were transcriptionally fused to *lacZ* and inserted into the *amyE* locus. We then assayed β-galactosidase activity during growth and sporulation. In agreement with the complementation experiments, we found that only the fragments of DNA that extended into *spoIIIAF* were able to drive production of β-galactosidase (Fig. 1A). In these strains, β-galactosidase activity began to accumulate about 1.5 h after the onset of sporulation, and maximum activity was reached approximately 2.5 h later (Fig. 1A). This temporal pattern of β-galactosidase activity was similar to that observed when $P1_{spoIIIA}$ was fused to *lacZ* (Fig. 1B). However, the promoter within *spoIIIAF*, $P2_{spoIIIA}$, produced twice as much β-galactosidase activity as $P1_{spoIIIA}$ (Fig. 1B).

To more precisely locate $P2_{spoIIIA}$, we examined the effect of sequential deletions from the 3′ end of *spoIIIAF* on β-galactosidase activity (Fig. 2). A fragment that included the first 415 bp of *spoIIIAF* directed transcription of *lacZ* and produced as much β-galactosidase activity as the 800-bp fragment. Sequential deletions were then made from the 5′ end of *spoIIIAF*, and we found that a fragment containing only about 200 bp of *spoIIIAF* directed expression of *lacZ* at the same level as the 800-bp fragment (Fig. 2). Therefore, the new promoter $P2_{spoIIIA}$ was located between bp 200 and 415 of *spoIIIAF*.

σ^E directs transcription from $P2_{spoIIIA}$. SpoIIIAH accumulates in the mother cell (1), and transcriptional array data indicate that its transcription is dependent on the mother cell-specific sigma factor σ^E (8, 19). Therefore, we examined the effects of mutations in genes responsible for both early and late mother cell gene expression on the activity of $P2_{spoIIIA}$. The early transcriptional regulators GerR and SpoIIID and secondary sigma factor σ^E and the late transcription factor GerE

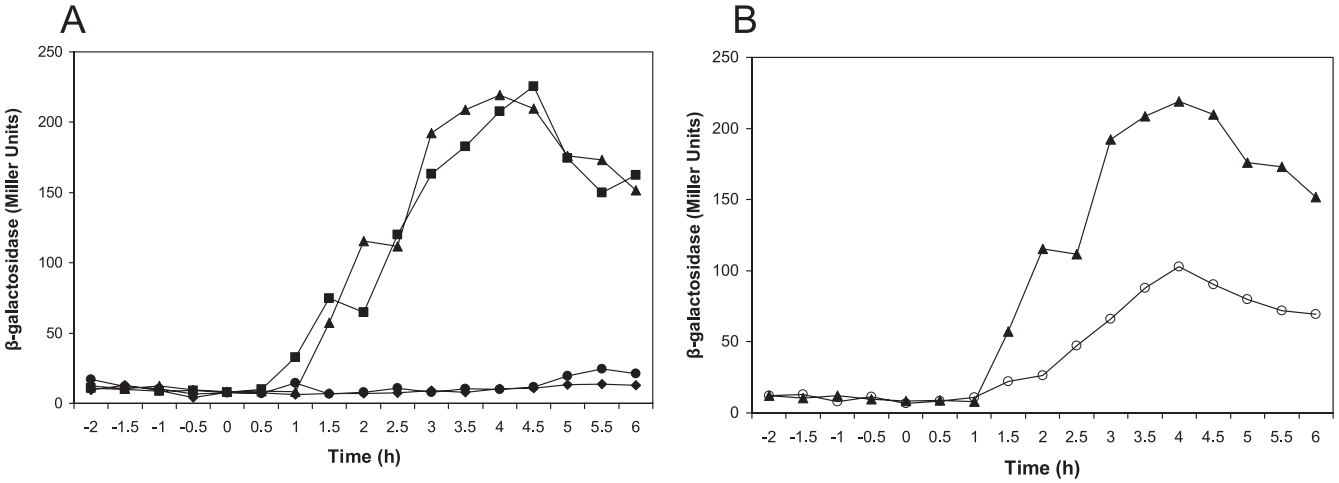

FIG. 1. Expression of *spoIIIA-lacZ* fusions. (A) β-Galactosidase accumulation in cultures of strains containing various regions of the *spoIIIA* locus, including *spoIIIAH* (◆), *spoIIIAGH* (●), *spoIIIAFGH* (▲), and *spoIIIA′EFGH* (■), transcriptionally fused to *lacZ*. (B) Comparison of $P1_{spoIIIA}$ and $P2_{spoIIIA}$ activities. β-Galactosidase activity was measured in strains containing $P1_{spoIIIA}$ (○) and *spoIIIAFGH*, which contains $P2_{spoIIIA}$ (▲), transcriptionally fused to *lacZ*. The data are the averages of three replicates. The *x* axis indicates the time (in hours) before and after the onset of sporulation (zero time).

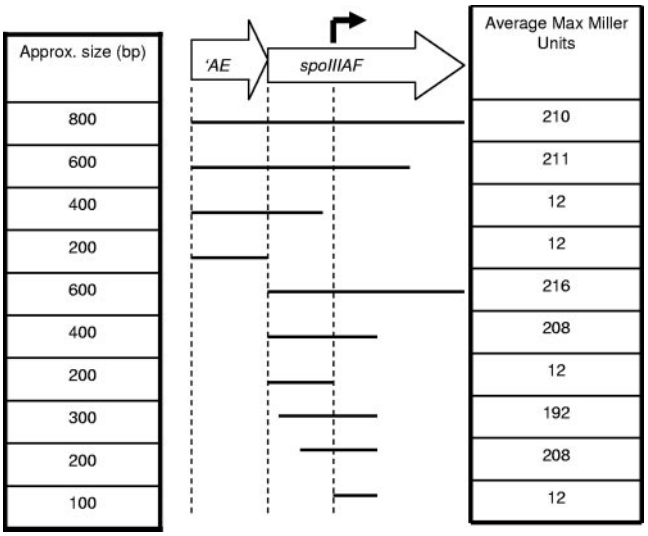

FIG. 2. Deletion analysis of the $P2_{spoIIIA}$ region. The large arrows indicate the region of the *spoIIIA* locus that contains $P2_{spoIIIA}$. The solid lines show the regions of the *spoIIIA* locus (approximate sizes are indicated on the left) that were transcriptionally fused to *lacZ* for β-galactosidase assays. The maximum accumulation of β-galactosidase for strains containing the fusion is indicated on the right (averages of three replicates).

and secondary sigma factor σ^K were inactivated by insertion of antibiotic cassettes. The effects of these mutations on expression of $P1_{spoIIIA}$-*lacZ* and $P2_{spoIIIA}$-*lacZ* were monitored during sporulation. Both promoters were found to be regulated by the same factors. σ^E was solely responsible for transcription of the promoters since very little β-galactosidase accumulated in the *sigE* mutant, and SpoIIID was responsible for repression of the promoters; in fact, in the absence of SpoIIID, twice as much β-galactosidase was produced from $P2_{spoIIIA}$ (Fig. 3 and data not shown). There were no significant effects on β-galactosidase activity when GerR, GerE, or σ^K was absent (Fig. 3).

We used primer extension to determine the transcription start site of $P2_{spoIIIA}$. RNA was harvested 4 h after the onset of sporulation from cultures of wild-type, Δ*sigE*, and Δ*spoIIID* strains harboring the $P2_{spoIIIA}$-*lacZ* fusion at *amyE*. We found that the 5′ end of the major transcription product mapped to bp 289 of *spoIIIAF* (Fig. 4). The effects on $P2_{spoIIIA}$ transcription of the mutations in *sigE*, which encodes σ^E, and *spoIIID* were the same as those seen in the β-galactosidase assays (Fig. 4); essentially, there was no transcript in the *sigE* deletion strain and there was about twice as much transcript in the *spoIIID* deletion strain.

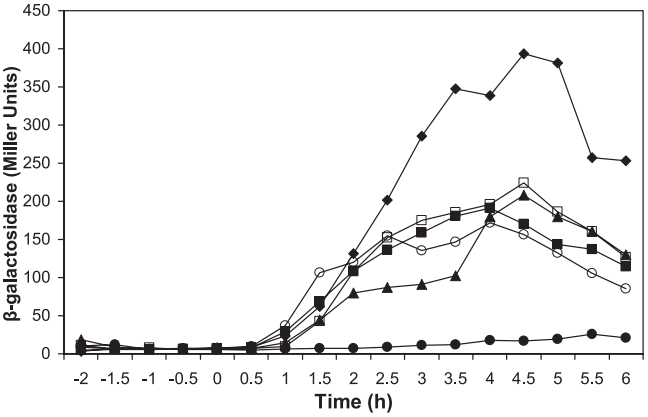

FIG. 3. Regulation of $P2_{spoIIIA}$: expression of a $P2_{spoIIIA}$-*lacZ* fusion in the wild type (▲) and mutant strains, including *gerR* (■), *spoIIID* (◆), *sigE* (●), *gerE* (□), and *sigK* (○) mutants. The data are averages of three replicates.

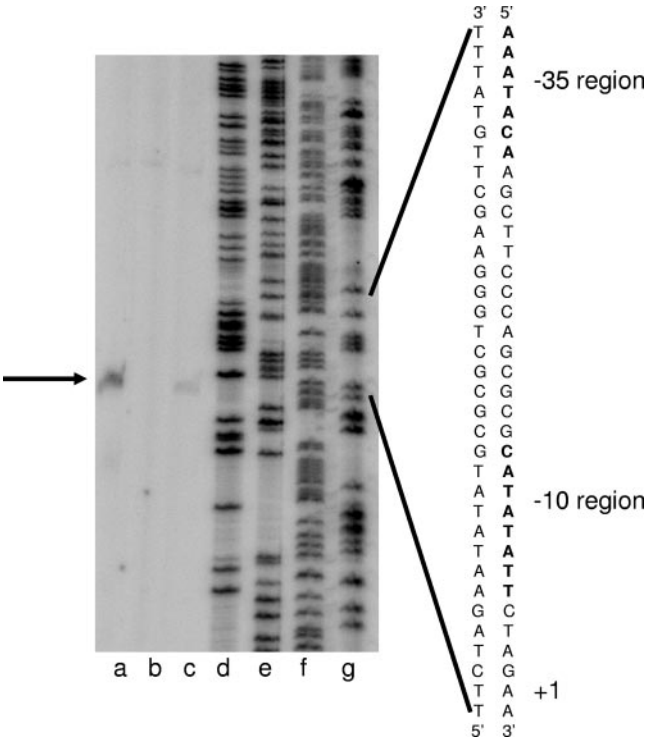

FIG. 4. Primer extension of analysis $P2_{spoIIIA}$. Total RNA was isolated 4 h after the onset of sporulation from three *B. subtilis* strains (Δ*spoIIID* [lane a], Δ*sigE* [lane b], and wild type [lane c]) harboring a $P2_{spoIIIA}$-*lacZ* fusion at *amyE*. The primer extension product and putative transcription start point are indicated by an arrow on the left and by +1 on the right. The −35 and −10 regions are also indicated to the right of the DNA sequence of the expanded region. Lanes d, e, f, and g contained the dideoxy sequencing reactions for G, A, T, and C, respectively; the same primer was used for sequencing and for primer extension.

Inspection of the sequence upstream from the putative $P2_{spoIIIA}$ start point of transcription revealed the sequences 5′AAATACA and 5′CATATATT (Fig. 5) in the −35 and −10 regions, respectively, that are similar to the consensus sequences in the −35 and −10 regions of σ^E-dependent promoters (7), especially in the most highly conserved sequence in the −35 region (ATA) and all of the −10 region. Since transcription from $P2_{spoIIIA}$ was dependent on σ^E (Fig. 3 and 4), we expected that the highly conserved −10 and −35 region sequences would play important roles in promoter activity if the 5′ end of the transcript indicated in our primer extension assays represented the actual start point of transcription. Therefore, we examined the effects of point mutations in the conserved sequences. The critical T residue in the −35 region (position −30) was changed to the nonconsensus base C, and the CA nucleotide pair in the −10 region (positions −12 and

FIG. 5. Sequence of the $P2_{spoIIIA}$ region. The nontranscribed strand encompassing $P2_{spoIIIA}$ from *B. subtilis* (*B. s.*), *Oceanobacillus iheyensis* (*O. i.*), *Bacillus cereus* (*B. c.*), *Bacillus thuringiensis* (*B. t.*), *Bacillus anthracis* Ames (*B. a.* A.), *Bacillus licheniformis* (*B. l.*), and *Geobacillus thermodenitrificans* (*G. t.*) is shown. The −35 and −10 regions are indicated by boldface type. The start point of transcription is indicated by +1, and positions −30 and −12 are indicated above the sequence. The underlined sequence is one potential SpoIIID binding site in which five of eight base pairs match the consensus sequence (8).

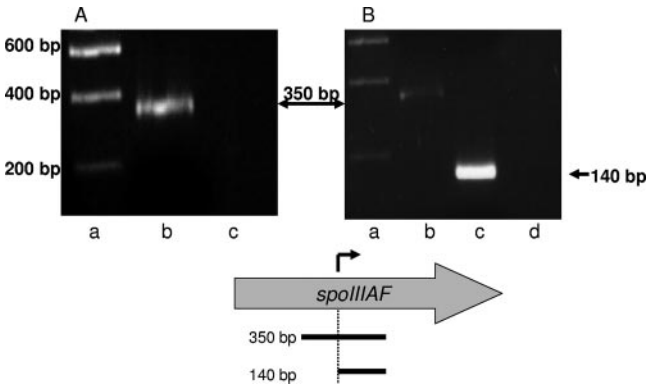

FIG. 6. RT-PCR analysis of transcripts in the $P2_{spoIIIA}$ region. RT-PCR was performed using two different forward primers in order to compare the amounts of transcript generated by $P1_{spoIIIA}$ alone and by $P1_{spoIIIA}$ and $P2_{spoIIIA}$ together. Each panel shows the ethidium bromide-stained DNA after electrophoresis in agarose. Lane a contained molecular weight standards in both panels. Lane b in both panels contained the 350-bp RT-PCR product of the transcript from $P1_{spoIIIA}$, whereas lane c in panel B contained the 140-bp RT-PCR product from transcripts originating from both $P1_{spoIIIA}$ and $P2_{spoIIIA}$. Lane c in panel A and lane d in panel B contained the products from control reactions in which no reverse transcriptase was added. Below panels A and B is a map of *spoIIIAF*. The arrow above *spoIIIAF* and the dotted vertical line indicate the $P2_{spoIIIA}$ start point of transcription. The horizontal lines below *spoIIIAF* indicate the 350- and 140-bp cDNA amplified from the $P1_{spoIIIA}$ and $P2_{spoIIIA}$ transcripts, respectively.

−11) was mutated to the nonconsensus nucleotide pair GT (Fig. 5). Both of these mutations caused complete inactivation of $P2_{spoIIIA}$ (data not shown).

The effects of mutations in the −10 and −35 regions of $P2_{spoIIIA}$ demonstrated that this promoter was responsible for the σ^E-dependent expression of the *lacZ* fusions described above, but we also tested whether this promoter was responsible for the expression of *spoIIIAG* and *spoIIIAH* at the *amyE* locus in the complementation experiments described above. We inserted the *spoIIIAFGH* fragment containing the −10 point mutations into the *amyE* locus and tested its ability to complement the *spoIIIAG* and *spoIIIAH* deletion mutants. We found that the mutant allele did not complement the sporulation defect in these strains (Table 4). Therefore, $P2_{spoIIIA}$ is required for expression of *spoIIIAG* and *spoIIIAH* in these strains.

$P1_{spoIIIA}$ plays no role in *spoIIIAG* and *spoIIIAH* expression. All of our results are consistent with the hypothesis that σ^E directs transcription from the $P2_{spoIIIA}$ promoter and that this transcription probably is sufficient for *spoIIIAG* and *spoIIIAH* expression. $P2_{spoIIIA}$ is located within *spoIIIAF*; therefore, it seemed likely that transcription initiated at $P1_{spoIIIA}$, which is required for *spoIIIAF* expression, would continue through $P2_{spoIIIA}$ and into *spoIIIAG* and *spoIIIAH*. We used RT-PCR to determine whether this read-through occurred. RNA was isolated from wild-type strain JH642 4 h after the onset of sporulation, and RT-PCR was performed for two regions near $P2_{spoIIIA}$: a 350-bp region that extended 180 bp upstream of $P2_{spoIIIA}$ to 150 bp downstream and a 140-bp region immediately downstream of $P2_{spoIIIA}$. After 30 cycles, the PCR products were electrophoresed on a 1.5% agarose gel. We detected the 350-bp product indicative of the read-through transcript (Fig. 6A). The 140-bp RT-PCR product that resulted from transcription from both $P1_{spoIIIA}$ and $P2_{spoIIIA}$ was much more abundant than the 350-bp product that represented transcription from only $P1_{spoIIIA}$ (Fig. 6B).

Although our complementation studies, in which we expressed *spoIIIAG* and *spoIIIAH* at an ectopic location, indicated that $P2_{spoIIIA}$ was necessary and sufficient for the expression of *spoIIIAG* and *spoIIIAH*, the RT-PCR results showing that transcripts from $P1_{spoIIIA}$ read into *spoIIIAG* raised the possibility, which was unlikely, that transcription originating from $P1_{spoIIIA}$ of *spoIIIAG* and *spoIIIAH* at the *spoIIIA* locus may enhance their expression and thus affect sporulation. Therefore, we isolated strains with Campbell-type insertions that would separate *spoIIIAG* and *spoIIIAH* from $P1_{spoIIIA}$ (Fig. 7A). A strain harboring the insertion in *spoIIIAF*, CGB175, exhibited almost wild-type levels of sporulation (99% of the wild-type levels). We also examined the effect of this Campbell-type insertion on expression of a σ^G-dependent *sspE-lacZ* fusion and found no effect on its expression, indicating that there were no subtle effects on σ^G activation (data not shown). As a control for these experiments, we also isolated a strain with an insertion in *spoIIIAE*, which resulted in separation of $P1_{spoIIIA}$ from *spoIIIAF* and downstream genes (Fig. 7B). This insertion reduced sporulation to 0.05% of that seen with a wild-type strain, indicating that the inserted sequences blocked transcription. Evidently, transcripts from $P1_{spoIIIA}$ read into *spoIIIAG* and *spoIIIAH* since they traverse $P2_{spoIIIA}$; however, they are not necessary to support sporulation. We also used the Campbell insertion technique to search for other potential promoters in the *spoIIIA* locus, but strains bearing such insertions in *spoIIIAD*, *spoIIIAC*, and *spoIIIAB* were sporulation defective (the sporulation efficiencies were 0.03 to 0.07% of the wild-type sporulation efficiencies), indicating that there are no other promoters (data not shown). This, along with our previ-

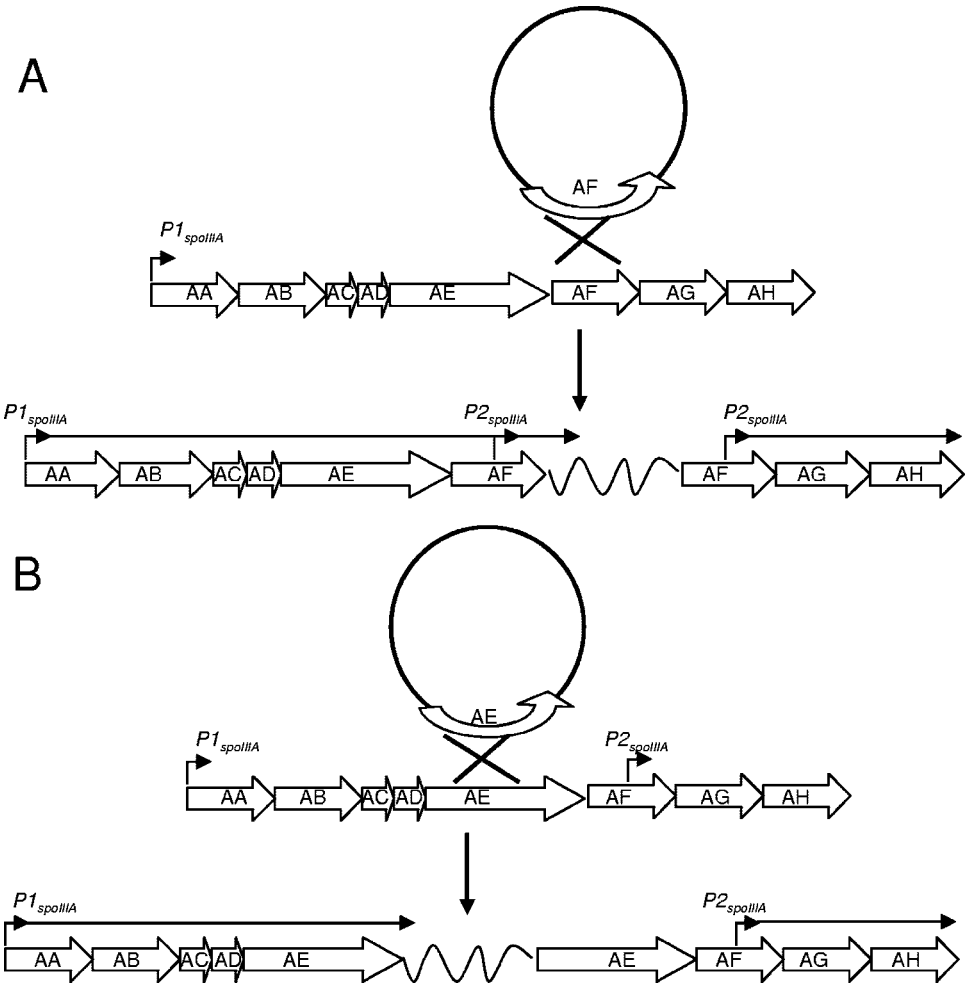

FIG. 7. Separation of *spoIIIAG* and *spoIIIAH* from $P1_{spoIIIA}$: models of the Campbell-type integration of plasmids into the *spoIIIA* locus. (A) Model in which a nonreplicating circular plasmid carrying *spoIIIAF* integrates into the *spoIIIA* locus by a single homologous recombination event. A horizontal arrow indicates that transcription from $P1_{spoIIIA}$ proceeds uninterrupted through *spoIIIAF*, while *spoIIIAG* and *spoIIIAH* are transcribed from $P2_{spoIIIA}$. (B) Model in which a nonreplicating circular plasmid carrying *spoIIIAE* integrates into the *spoIIIA* locus by a single homologous recombination event. A horizontal arrow indicates that transcription from $P1_{spoIIIA}$ is interrupted by plasmid sequences (wavy line) before entering *spoIIIAF*, resulting in a sporulation-defective phenotype. The intervening plasmid DNA is represented by a wavy line in both panels and is not to scale.

ous complementation results (Fig. 1A and B), indicated that transcription of *spoIIIAG* and *spoIIIAH* from $P2_{spoIIIA}$ alone is sufficient for sporulation.

***$P2_{spoIIIA}$* is essential for sporulation.** The results of complementation tests indicated that transcription of *spoIIIAG* and *spoIIIAH* from $P2_{spoIIIA}$ is sufficient when these genes are located at the ectopic *amyE* locus. At the chromosomal *spoIIIA* locus, transcription of *spoIIIAH* from $P1_{spoIIIA}$ is not essential (1), so we predicted that transcription of both *spoIIIAG* and *spoIIIAH* from $P2_{spoIIIA}$ at the *spoIIIA* locus is essential for sporulation. Therefore, we isolated a strain (CGB225) containing two base pair substitutions at positions −12 and −11 of $P2_{spoIIIA}$ within the *spoIIIA* locus. These mutations resulted in a sporulation-deficient phenotype (0.025 to 0.7% of the wild-type sporulation level) and also reduced the expression of a σ^G-dependent *sspE-lacZ* fusion (CGB226) to levels that were less than 30% of the wild-type levels (data not shown). We noted that the mutations in $P2_{spoIIIA}$ resulted in a change in the coding region of *spoIIIAF* by producing an alanine-to-glycine substitution in SpoIIIAF. However, this amino acid substitution was not responsible for the sporulation-defective phenotype since spore formation was complemented to wild-type levels by expression of *spoIIIAG* and *spoIIIAH* from the $P2_{spoIIIA}$ promoter at *amyE* in strain CGB227, a derivative of CGB225.

DISCUSSION

Our results indicate that the *spoIIIA* locus, which encodes eight genes, is transcribed from two promoters, $P1_{spoIIIA}$, which is located at the start of the locus, and $P2_{spoIIIA}$, which is located within the open reading frame of *spoIIIAF*. $P2_{spoIIIA}$ is required for transcription of *spoIIIAG* and *spoIIIAH*. RT-PCR analysis showed that transcripts from $P1_{spoIIIA}$ probably read through *spoIIIAG* and *spoIIIAH*, but the $P1_{spoIIIA}$-dependent transcripts may not produce enough *spoIIIAG* and *spoIIIAH*

products. This conclusion follows from our observations that $P2_{spoIIIA}$-*lacZ* was expressed at twice the levels of $P1_{spoIIIA}$-*lacZ* (Fig. 1B) and that expression of a $P1_{spoIIIA}$-*spoIIIAH* fusion was not sufficient to complement the sporulation defect of a mutant *spoIIIAH* strain (Table 4). Furthermore, expression of *spoIIIAG* and *spoIIIAH* from $P2_{spoIIIA}$ is sufficient to support sporulation, since $P2_{spoIIIA}$-driven expression of *spoIIIAG* and *spoIIIAH* complements from an ectopic location (Table 4), and an insertion physically separating *spoIIIAG* and *spoIIIAH* from $P1_{spoIIIA}$ had no detectable effect on sporulation efficiency or on the timing of σ^G activation (data not shown). Moreover, mutation of $P2_{spoIIIA}$ at the *spoIIIA* locus caused a sporulation-defective phenotype that could be complemented by expression of *spoIIIAG* and *spoIIIAH* at the *amyE* locus.

There are many examples of complex operons containing more than one promoter in both gram-positive and gram-negative bacteria (3, 14, 16, 24). However, the most common reason for having more than one promoter for an operon is to ensure its expression under different growth conditions or requirements. The promoters of these operons are usually recognized by different sigma factors or may require other types of different transcription factors for expression. Therefore, a potential explanation for the necessity of a second promoter within the *spoIIIA* locus could be that $P1_{spoIIIA}$ and $P2_{spoIIIA}$ are differentially regulated. However, this possibility is unlikely because transcription from both promoters appears to be directly dependent on σ^E and the activities of both promoters are repressed by SpoIIID. We found at least two potential SpoIIID binding sites centered at position −22 relative to the $P2_{spoIIIA}$ transcription start site (bp 301 of *spoIIIAF*) (Fig. 5) and at position 11 (bp 268 of *spoIIIAF*) (not shown). These conserved sequences and the elevated expression from $P2_{spoIIIA}$ that we observed in the *spoIIID* mutant (Fig. 3 and 4) are consistent with a model in which SpoIIID directly represses $P2_{spoIIIA}$ activity, as is the case for $P1_{spoIIIA}$ (8, 13). Therefore, it is unlikely that the two promoters in the *spoIIIA* locus are differentially regulated; rather, it is likely that the primary purpose of $P2_{spoIIIA}$ has to do only with increasing the expression of the last two genes of the locus.

Although the reason that a second promoter in the locus, $P2_{spoIIIA}$, is required is not entirely clear, this promoter may be a conserved feature within the *spoIIIA* locus of other spore formers (Fig. 5). The hypothesis that the second promoter in the locus, $P2_{spoIIIA}$, is required because higher levels of SpoIIIAG and SpoIIIAH than of the upstream *spoIIIA* products are required for sporulation led us to speculate that SpoIIIAG and SpoIIIAH play different types of roles in sporulation than the other members of the locus. This idea is supported by topology predictions of SpoIIIAG and SpoIIIAH that show that these proteins are more similar to one another than to any other protein encoded by the *spoIIIA* locus (4, 20, 22). SpoIIIAH is known to recruit other proteins involved in intercellular signaling to the septum separating the mother cell and forespore (6). SpoIIIAH has also been postulated to help drive the engulfment of the forespore (2). The SpoIIIA proteins encoded by upstream genes are also essential for spore formation and, like SpoIIIAH and SpoIIIAG, are required for activation of σ^G (15). However, these proteins, SpoIIIAA to SpoIIIAF, may act catalytically or at least at lower stoichiometries than SpoIIIAG and SpoIIIAH.

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