Organization and Regulation of an Operon That Encodes a Sporulation-Essential Sigma Factor in *Bacillus subtilis*

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Deletion of *sigE*, the structural gene for the sporulation-induced RNA polymerase sigma factor, σ^E , prevented endospore formation by *Bacillus subtilis*. The effects of integration of plasmids into the *sigE* region of the chromosome and the use of complementation analyses demonstrated that *sigE* is part of an operon that includes a promoter-proximal gene, *spoIIA*, that is essential for sporulation. Gene fusions to the promoter of this operon, *spoIIG*, demonstrated that transcription from this promoter is induced at the beginning of sporulation and is dependent on several *spo0* genes.

Bacillus subtilis is capable of a remarkably complex differentiation culminating in the production of an endospore. This differentiation requires the expression of approximately 50 genetic loci that have been identified by mutations that block endospore formation at any one of several distinct morphological stages (for a review, see reference 12). Several of these loci are not transcribed until after the initiation of endospore formation (20, 22), and attempts to reconstruct their transcription in vitro has led to the discovery of several forms of RNA polymerase that differ only in their sigma subunits and, thereby, their specificity for promoter recognition (3, 7, 8).

Five forms of RNA polymerase are present in vegetative cells. Two hours after the initiation of sporulation, another sigma factor (σ^E , formerly σ^{29}) is synthesized (7). RNA polymerase that contains this sigma factor, $E\sigma^E$, is the predominant form found between hours 2 and 4 of sporulation and therefore may be responsible for most of the transcription during this period. The promoters of several genes, at least one of which is essential for sporulation (*spoIID*), can be used in vitro by $E\sigma^E$ (19, 20). Transcription of these genes in vivo is dependent on the synthesis of σ^E (19, 20); therefore, RNA polymerase that contains this sigma factor appears to be necessary for the expression of some genes during this period of development.

Mutations at the *spoIIG* locus block endospore formation. The wild-type allele of this locus has been cloned on a 1.1-kilobase (kb) *Pst*I DNA fragment from *B. subtilis*, which when carried in plasmid pGSIIG11, complements the sporulation-defective phenotype of several *spoIIG* mutants (23). Nucleotide sequence analysis indicated that this DNA fragment encodes a single large polypeptide with a predicted mass of 27,652 daltons (23). Trempy et al. (24) concluded that this DNA fragment encodes σ^E , since both *Escherichia coli* and *B. subtilis* that contained plasmid pGSIIG11 produced polypeptides that reacted with a monoclonal antibody that was highly specific for σ^E . Analysis of the N-terminal amino acid sequence of σ^E has confirmed this conclusion (W. G. Haldenwang, personal communication).

Hybridization with DNA of the pGSIIG11 plasmid has indicated that RNA complementary to this region accumulates only after the initiation of sporulation (24). The accumulation of an RNA that encodes this sporulation-essential sigma factor may be due to an increased rate of specific

transcription at the beginning of sporulation. To investigate this possibility, we located the promoter for the *sigE* gene and found that it is activated at the beginning of sporulation. We also found that the *spoIIG* transcriptional unit contains at least two genes that are essential for sporulation, *sigE*, which encodes σ^E , and a promoter-proximal gene that we refer to as *spoIIA*.

MATERIALS AND METHODS

Bacterial strains. The *B. subtilis* strains used here are listed in Table 1.

General cloning procedures. Restriction endonuclease cleavage reactions and ligations were done in accordance with the specifications of the manufacturers (Amersham Corp. and Boehringer Mannheim Biochemicals), except that ligations of blunt-end DNA fragments were done in the presence of 5% (wt/vol) polyethylene glycol. Most of the DNA fragments used for cloning were purified by electrophoresis on low-melting-point agarose. After ligation, recombinant plasmids were transformed into *E. coli* HB101 (13) or TB1 (obtained from Bethesda Research Laboratories, Inc.) and bacteriophage M13 recombinants were transfected into *E. coli* 71.18 (26). Plasmid transformants were selected by growth on LB plates containing 60 μ g of ampicillin per ml, 10 μ g of tetracycline per ml, or 15 μ g of chloramphenicol per ml, and M13 recombinants were identified as white plaques on plates containing 40 μ g of 5-bromo-4-chloro-3-indolyl- β -D-galactoside. In all instances, the resulting plasmids or phage replicative-form DNAs were characterized by restriction site mapping.

Construction of *sigE* deletion. The 2.3-kb fragment containing the *erm* gene from *B. subtilis* plasmid pTV39, a derivative of pTV8 (27) provided by P. Youngman, was removed by cutting the plasmid with *Bam*HI and *Sal*I. This fragment was cloned into pUC18 (26) between the *Bam*HI and *Sal*I sites. The recombinant plasmid (pUC18-*erm*) was then cut with *Hind*III and *Eco*RI, and the 2.3-kb fragment containing the *erm* gene was cloned into plasmid pJH101 (6) between the *Hind*III and *Eco*RI sites to produce plasmid pJH101E.

The 1.1-kb *Pst*I DNA fragment from pGSIIG11 (23) that contained the coding sequence for *sigE* was digested to completion with *Dde*I. The 170-base-pair (bp) *Dde*I-*Pst*I DNA fragment containing the promoter-distal end of this fragment was treated with S1 nuclease to generate blunt ends and cloned into the *Eco*RV site of pJH101E. The orientation of the insert was determined by cleavage of the recombinant

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TABLE 1. *B. subtilis* strains

Strain	Relevant genotype and derivation ^a
JH642	<i>trpC2 phe-1</i> (28)
BS50	<i>spoIIG41</i> (24)
EU8701	JH642 Δ <i>sigE::erm</i>
EU8711	JH642 Ω pTK1
EU8712	JH642 Ω pTK2
EU8713	JH642 Ω pTK3
EU8714	JH642 Ω pTK4
EU8715	JH642 Ω pTK5
EU8716	JH642 Ω pTK6
EU8717	JH642 Ω pTK7
EU8718	JH642 Ω pTK8
EU8719	JH642 Ω pTK9
EU8720	JH642 Ω pTK10
EU8721	JH642 Ω pTK11
EU8722	JH642 Ω pTK12
EU8723	JH642 Ω pTK13
EU8724	JH642 Ω pTK14
EU8725	JH642 Ω pTK15
PY222	Prototroph, pTV17 (27)
PY105	<i>trpC2</i> SP β :: <i>c2 del2::Tn917</i> (27)
EU8726	1.1 (4) <i>spoIIAC1</i> SP β IIG- <i>lacZ</i>
EU8727	JH646 (28) <i>spo0A12</i> SP β IIG- <i>lacZ</i>
EU8728	ZB369 (29) <i>spo0A abrB</i> SP β IIG- <i>lacZ</i>
EU8729	JH648 (28) <i>spo0B136</i> SP β IIG- <i>lacZ</i>
EU8730	JH649 (28) <i>spo0F221</i> SP β IIG- <i>lacZ</i>
EU8731	JH693 (28) <i>spo0J93</i> SP β IIG- <i>lacZ</i>
EU8732	JH651 (28) <i>spo0H81</i> SP β IIG- <i>lacZ</i>
EU8733	IS233 (25) Δ <i>spo0H</i> SP β IIG- <i>lacZ</i>
EU8734	ML1 ^b <i>sigB</i> SP β IIG- <i>lacZ</i>
EU8735	EU8701 SP β <i>sigE</i>
EU8736	EU8701 SP β <i>gis</i>
EU8737	EU8725 SP β <i>sigE</i>
EU8738	EU8725 SP β <i>gis</i>
EU8739	EU8719 SP β <i>sigE</i>
EU8740	EU8719 SP β <i>gis</i>
EU8741	EU8723 SP β <i>sigE</i>
EU8742	EU8723 SP β <i>gis</i>
EU8743	JH642 SP β IIG- <i>lacZ</i>
EU8744	EU8701 SP β IIG- <i>lacZ</i>
EU8745	BS50 SP β <i>sigE</i>
EU8746	BS50 SP β <i>gis</i>

^a If a reference is not given the strain was isolated in this study as a derivative of the strain indicated (i.e., strain EU8711 was derived by transformation of strain JH642 with the integrational plasmid pTK1, and strain EU8735 is an SP β *sigE* lysogen of strain EU8701).

^b M. Igo, M. Lampe, C. Ray, W. Schafer, C. P. Moran, Jr., and R. Losick, Mol. Gen. Genet., in press.

plasmid (pJH101E3) with *ScaI*, which cleaved the 170-bp insert 42 bp from its promoter-distal end.

Plasmid pTK1 (described below) was opened at the *EcoRI* site and subjected to brief Bal-31 exonuclease (International Biotechnologies Inc.) treatment followed by ligation with *EcoRI* linkers. Clones were screened by restriction enzyme digestion and gel electrophoresis. The clone containing 160 bp of the promoter-proximal end of the 1.1-kb *PstI* fragment was used to transform *B. subtilis* JH642 to chloramphenicol resistance. The plasmid integrated into the chromosome via a Campbell-like mechanism (see below). Chromosomal DNA from a transformant was cut with *EcoRV*, ligated at 150 μ g of DNA per ml to recircularize the plasmid, and used to transform *E. coli* HB101. Transformants were selected for growth in the presence of 60 μ g of ampicillin per ml. Two hundred clones were screened by colony hybridization for the presence of the upstream *spoIIG* sequences. The one positive clone was cut with *EcoRI*, and the 2,160-bp *EcoRI* DNA fragment from the region upstream from the *sigE* gene

was then cloned into pJH101E3 at the *EcoRI* site. The orientation of this insert was determined by cleavage of the recombinant plasmid, pJH1015E3, with *PstI*, which cleaved the insert 160 bp from the promoter-proximal end.

Plasmid pJH1015E3 was cut with *BglI*, which cut the plasmid three times but did not cut within the cloned sequences. The linearized DNA was transformed into competent *B. subtilis* JH642, and transformants were selected by growth in the presence of erythromycin (1 μ g/ml) and lincomycin (25 μ g/ml).

Sporulation tests. The number of heat-resistant spores per milliliter was determined after 24 h of growth in liquid DS medium (DSM) (28) by heating 1 ml of the culture at 80°C for 10 min. Appropriate dilutions of the cultures were plated before and after heat treatment. Sporulation phenotypes were determined by pigment production on DSM plates (28), on which spore-forming colonies produce a brown pigment, whereas nonsporulating colonies remain white.

Construction of integrational plasmids. pTK1 was constructed in two steps. First, the 200-bp *PstI*-*HincII* DNA fragment from pGSIIG11 (23) was cloned between the *PstI* and *SmaI* sites of M13mp19 (26). The 231-bp *HindIII*-*EcoRI* DNA fragment that contained this region in the resulting M13mp19 derivative was cloned between the *HindIII* and *EcoRI* sites of pJH101 (6) (see Fig. 4 and 5 for restriction site maps of the *spoIIG* region).

pTK2 was also constructed in two steps. The 500-bp *PstI*-*HindIII* fragment from pGSIIG11 was cloned between the *PstI* and *HindIII* sites in M13mp18. The 518-bp *HindIII*-*BamHI* fragment that contained this region in the M13 derivative was cloned between the *HindIII* and *BamHI* sites of pJH101.

Again, two steps were used to construct pTK3. The 300-bp *HincII*-*HindIII* fragment from pGSIIG11 was cloned between the *HincII* and *HindIII* sites of M13mp19. The 315-bp *HindIII*-*BamHI* fragment that contained this region in the M13 derivative was cloned between the *HindIII* and *BamHI* sites of pJH101.

Two similar steps were used to construct pTK4. The 430-bp *PstI*-*HindIII* fragment was cloned between the *PstI* and *HindIII* sites of M13mp18. The 448-bp *HindIII*-*BamHI* fragment that contained this region in the M13 recombinant was cloned into pJH101 between the *HindIII* and *BamHI* sites.

pTK5 was constructed by cutting chromosomal DNA from *B. subtilis* EU8711, which contained an integrated copy of pTK1, with *EcoRV*, followed by circularization of this DNA by ligation of a dilute solution (150 μ g of DNA per ml). This ligation mixture was transformed into *E. coli*, and transformants were selected as described above by growth in the presence of 60 μ g of ampicillin per ml.

pTK6 was constructed by cloning the 2,200-bp *EcoRI*-*EcoRV* fragment from pTK5 between the *EcoRI* and *EcoRV* sites of pJH101. pTK7 was constructed by adding the 2,200-bp *EcoRI* fragment from pTK5 into the *EcoRI* site of pJH101. pTK8 was constructed by cloning the 1,500-bp *EcoRI*-*PstI* DNA fragment from pTK7 into pJH101 between the *EcoRI* and *PstI* sites. pTK9 was constructed by cloning the 500-bp *PstI* fragment from pTK7 into the *PstI* site of pJH101. pTK10 was constructed by cloning the 1-kb *EcoRI*-*TaqI* fragment from pTK8 between the *EcoRI* and *ClaI* sites of pJH101. pTK11 was constructed by cloning the 480-bp *TaqI*-*PstI* fragment from pTK8 between the *ClaI* and *PstI* sites of pJH101.

To construct pTK12, the 480-bp *TaqI*-*PstI* fragment from pTK11 was gel purified and digested with *AhaIII*, and the

100-bp *AhaIII*-*PstI* fragment was cloned between the *ScaI* and *PstI* sites of pJH101. To construct pTK13, the 480-bp *TaqI*-*PstI* fragment from pTK11 was gel purified and digested with *AhaIII*, and the 160-bp *AhaIII* fragment was cloned into pJH101 at the *EcoRV* site. To construct pTK14, the 480-bp *TaqI*-*PstI* DNA fragment from pTK11 was gel purified and digested with *AhaIII*, and the 220-bp *TaqI*-*AhaIII* fragment was cloned between the *ClaI* and *EcoRV* sites of pJH101. pTK15 was constructed by cloning the 800-bp *AhaIII*-*EcoRI* fragment from pTK7 between the *EcoRV* and *EcoRI* sites of pJH101.

Construction of *B. subtilis* strains containing integrational plasmids. Each of the 15 integrational plasmids (see Fig. 4 and 5) was used to transform competent *B. subtilis* JH642, and chloramphenicol-resistant transformants were selected by growth on LB supplemented with 5 μ g of chloramphenicol per ml.

Construction and use of specialized transducing phages. Specialized transducing phages were constructed by the procedures described by Igo and Losick (9). In each instance, the fragment of DNA that was to be moved into the genome of *Bacillus* phage SP β was first cloned into pTV11 or pTV12 near the *cat* gene, between the two ends of Tn917 (9), by using homology-assisted transformation with pTV17 (9). The recombinant plasmids were then transformed into PY105, which contained prophage SP β *c2 del2::Tn917*. Recombination between the plasmid and the homologous regions in Tn917, which was located in the SP β prophage, resulted in insertion of *cat* and adjacent DNA into the prophage (Fig. 1 and 2). Plasmid pTK1217 (Fig. 1) was constructed by cloning the 1.1-kb *PstI* DNA fragment from pGSIIG11 that contained *sigE* into the *BamHI* site of pTV12 after the addition of linker DNA to the *PstI* fragment. This plasmid was used to produce SP β *c2 del2::Tn917::cat11 sigE* (SP β sigE). A similar plasmid that contained the 1.1-kb fragment in the opposite orientation was used to produce SP β *c2 del2::Tn917::cat11 gis*(SP β gis). A specialized transducing phage that carried the *spoIIIG* promoter fused to *lacZ* was constructed by cloning the 480-bp *TaqI*-*PstI* DNA fragment which contained the promoter next to a 3-kb *BamHI*-*AhaIII* fragment that contained the promoterless *lacZ* coding sequence from pTV32 (27). This promoter-*lacZ* cassette was cloned into pTV17 to produce pTK1117 (Fig. 2). The promoter-*lacZ* fusion and *cat* were then recombined onto SP β *c2 del2::Tn917* to produce SP β *c2 del2::Tn917::cat11 spoIIIG-lacZ* (SP β IIG-*lacZ*) (Fig. 2).

DNA sequencing. The 217-bp *TaqI*-*AhaIII* fragment from plasmid pTK11 that contained the promoter for *spoIIIG* was cloned between the *AccI* and *SmaI* sites of M13mp19. In addition, this fragment was cloned as a *HindIII* (6 bp past the *TaqI* site in pTK11)-*AhaIII* fragment between the *HindIII* and *HincII* sites of M13mp18. The sequences of both strands of this insert were then determined by the dideoxy sequencing protocol (21).

β -Galactosidase assays. β -Galactosidase assays were done by the method of Miller (15). *B. subtilis* strains containing prophage SP β IIG-*lacZ* were grown to an optical density at 600 nm (OD₆₀₀) of 0.5 in TSS minimal medium (28) containing 5 μ g of chloramphenicol per ml (or 1 μ g of erythromycin per ml for strain EU8734) and the appropriate amino acids. At that time (time zero), decoyinine (The Upjohn Co.) was added to a final concentration of 500 μ g/ml to induce sporulation. At hourly intervals, 1-ml cell culture samples were removed, pelleted, and washed in 50 mM Tris hydrochloride (pH 7.5). The cell pellets were stored at -70°C overnight. The cells were suspended in 1 ml of Z buffer (15),

FIG. 1. Construction of SP β sigE specialized transducing phage. Plasmid pTK1217 was transformed into strain PY105, which contained the prophage SP β *c2 del2::Tn917*. After induction, a specialized transducing phage that carried the *cat* gene was selected by transduction of strain JH642 to chloramphenicol resistance. The recombination events between Tn917 in the SP β *c2 del2::Tn917* prophage and the homologous regions of pTK1217 are shown. These crossovers recombined *sigE* and *cat* into the prophage DNA. The wavy arrows indicate the direction of *erm*, *cat*, and *sigE* transcription. Restriction enzyme cleavage sites: S, *SmaI*; B, *BamHI*; P, *PstI*.

and 2 drops of chloroform and 1 drop of 0.1% sodium dodecyl sulfate were added and mixed vigorously for 10 s on a tabletop vortex apparatus. The samples were placed at 28°C for 5 min, and then 200 μ l of *o*-nitrophenyl- β -D-galactopyranoside (4 mg/ml) was added. The reaction mixtures were incubated at 28°C for 1 h, at which time 0.5 ml of 1 M NaCO₂ was added to stop the reaction. The samples were then centrifuged for 5 min at 12 $\times$ *g* to pellet the cell debris, and the OD₄₂₀ of the supernatants was read. Miller units were calculated by the following formula: units = 1,000 $\times$ OD₄₂₀/(time $\times$ volume $\times$ OD₆₀₀).

Northern blot analysis. RNA was purified by centrifugation through CsCl by a modification of the procedure of McDonald et al. (14). *B. subtilis* JH642 was grown in DSM (28), and the cells were harvested at the mid-log phase and at 1 and 2 h after the end of exponential growth. The cell pellet was suspended in 3 ml of lysis buffer (100 mM Tris hydrochloride [pH 6.8], 2 mM EDTA, 3 mg of lysozyme per ml). After a 10-min incubation at 37°C, 0.3 ml of 10% sodium dodecyl sulfate was added, the samples were boiled for 5 min and placed on ice, and 300 μ l of 2 M KCl was added. After 30 additional min on ice, the samples were centrifuged at 8,000 rpm for 10 min in a Sorvall SS34 rotor. The volume of the supernatant was brought to 3 ml by the addition of 0.4 ml of 0.5 M EDTA (pH 8.0) and H₂O. This solution was centrifuged through CsCl as described previously (18). The RNA pellet was dissolved in 0.3 ml of 10 mM Tris hydrochloride (pH 6.8)–5 mM EDTA and extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1). The RNA was then precipitated twice by the addition of 0.25 M sodium acetate and 2.5 volumes of ethanol, and the precipitate was dissolved in 100 to 200 μ l of sterile water.

RNA was subjected to electrophoresis in formaldehyde-agarose (1.2%) gels as described previously (1). RNA samples (20 μ g) were mixed with 3 volumes of loading buffer [5 ml of formamide, 1.67 ml of formaldehyde, 200 μ l of 1 M

FIG. 2. Construction of *spoIIIG-lacZ* specialized transducing phage (SP β IIIG-*lacZ*). Plasmid pTK1117 was transformed into strain PY105, which contained the prophage SP β *c2 del2::Tn917*. After induction, a specialized transducing phage that carried the *cat* gene was selected by transduction of *B. subtilis* JH642 to chloramphenicol resistance. The recombination events between Tn917 in the SP β *c2 del2::Tn917* prophage and the homologous regions on pTK1117 are shown. These crossovers recombined *cat* and the *spoIIIG-lacZ* cassette into the prophage DNA. The wavy arrows indicate the direction of *erm*, *cat*, and *lacZ* transcription. Restriction enzyme cleavage sites: B, *Bam*HI; S, *Sma*I; P, *Pst*I; T, *Taq*I.

3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 7.0), 25 μ l of 400 mM EDTA, and 25 μ l of 2 M sodium acetate (pH 5.5), brought to 7.5 ml with H₂O] and heated at 65°C for 15 min before being loaded on the gel. The gel was subjected to electrophoresis at 30 V for 16 h.

The gel was rinsed in H₂O and incubated for 45 min at room temperature in 50 mM NaOH–10 mM NaCl, 45 min in 0.1 M Tris hydrochloride (pH 7.4), and 1 h in 20 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (pH 7.0). Transfer onto nitrocellulose (BA75) was performed in 20 $\times$ SSC for 4 h. The nitrocellulose was air dried and then baked in a vacuum oven at 80°C for 3 h.

The gel was prehybridized at 65°C for 4 h in 5 $\times$ SSC–5 $\times$ Denhardt solution (0.1% [wt/vol] Ficoll [Pharmacia Fine Chemicals], 0.1% polyvinyl pyrrolidone, 0.1% bovine serum albumin)–200 μ g of sonicated salmon sperm DNA–0.1% sodium dodecyl sulfate. Hybridization probes were made with oligodeoxynucleotides and the Klenow fragment of DNA polymerase by the method of Feinberg and Vogelstein (5). The probes were added to the prehybridization mix, incubated for 16 h at 65°C, and then washed for 2 h in 2 $\times$ SSC–0.1% sodium dodecyl sulfate and 1 h in 0.5 $\times$ SSC with several changes of buffer. After being air dried, the blots were exposed to X-ray film.

Promoter fusions to *xylE*. To construct a transcriptional gene fusion between the *sigE* promoter and the *xylE* gene, the 480-bp *Taq*I–*Pst*I DNA fragment containing the promoter for *sigE* was removed as a *Hind*III–*Bam*HI fragment from a pUC19 derivative containing this insert and cloned between the *Hind*III and *Bam*HI sites of pLC1 (18) to produce plasmid pTK480.

Plasmid pTK14, which contains the 217-bp *Taq*I–*Aha*III fragment of the *sigE* promoter was digested with *Eco*RI and *Bam*HI, and the promoter fragment was cloned between the

*Eco*RI and *Bam*HI sites of pLC4, a derivative of pLC1 (18) to produce plasmid pTK217. The *Bam*HI–*Hind*III fragment containing the first 500 bp of the 1.1-kb *Pst*I fragment from plasmid pTK4 was cloned between the *Hind*III and *Bam*HI sites of pLC4 to produce pTK500. pTK2200 was constructed by cloning the 2,200-bp *Eco*RI fragment from pTK7 into the *Eco*RI site of pLC1. The orientation of the 2,200-bp fragment was determined by digestion of the plasmid with *Pst*I. pTK800 is a pLC1 derivative that contains the 800-bp *Aha*III–*Eco*RI fragment from pTK15. In addition, it contains the 2.0-kb Ω fragment (17) containing transcriptional and translational stop signals immediately upstream from the *Aha*III–*Eco*RI fragment to prevent plasmid instability in *E. coli*.

Assays for catechol 2,3-dioxygenase (CatO₂ase) were done as described previously (18) with the following exceptions. Cells were grown in DSM containing 5 μ g of chloramphenicol per ml. Specific activities of CatO₂ase were determined spectrophotometrically at 30°C in a total volume of 3 ml, which contained 0.5 ml of cell extract, 2.5 ml of 100 mM potassium phosphate (pH 7.5), and 0.07 mM catechol.

RESULTS

Deletion of *sigE*. The best evidence that σ^E is necessary for sporulation is the observation that a mutation which maps within the 1.1-kb *Pst*I DNA fragment that includes the *sigE* gene (*spoIIIG55*) results in a sporulation-deficient phenotype and prevents the production of σ^E (24). The nature of this mutation is not known. We constructed a null allele of *sigE* in vitro by replacing the coding region of the gene with a DNA fragment from Tn917 that encodes resistance to erythromycin (*erm*). A linear DNA fragment that included the sequences which are contiguous with each end of the *sigE*

coding sequence, as well as the *erm* gene (plasmid pJH1015E3; see Materials and Methods), was used to transform *B. subtilis* JH642, and erythromycin-resistant transformants were selected. To confirm that the *sigE* coding region had been deleted in the transformant (EU8701), the chromosomal DNA of the transformant was cleaved with *EcoRI* and the resulting DNA fragments were separated by electrophoresis in an agarose gel. The fragments were transferred to nitrocellulose by ascending chromatography (15), and hybridization with several radiolabeled DNA fragments was used to detect the homologous *EcoRI* fragments from the transformed strain. The 1.1-kb *PstI* fragment that contains *sigE* (see Fig. 4) hybridized to a 10-kb *EcoRI* fragment from the parental strain JH642 (Fig. 3, lane a) and to two fragments (12 and 2.2 kb) from EU8701 (Fig. 3, lane b). This was expected since a new *EcoRI* site had been introduced near *erm* between the sequences that are located on each side of *sigE*. This result also demonstrated that the 10-kb *EcoRI* fragment in which *sigE* resides in JH642 had been disrupted. Hybridization with the 300-bp *HincII-HindIII* fragment from the *sigE* coding region (23) detected a 10-kb *EcoRI* fragment from JH642 (Fig. 3, lane c), but did not detect a fragment from EU8701 (Fig. 3, lane d), demonstrating that this region had been deleted. Hybridization with fragments from the regions immediately adjacent to the promoter-proximal and -distal ends of the *sigE* coding sequence detected the 10-kb *EcoRI* fragment from JH642 but only the 12- and 2-kb fragments, respectively, from EU8701 (data not shown). These hybridization analyses confirmed that the DNA fragment containing *erm* had inserted into the chromosome between the sequences at each end of *sigE* and the homologous sequences that had been cloned at each end of *erm*. They also confirmed that the insertion of *erm* resulted in the deletion of *sigE* coding sequences from the chromosome.

B. subtilis EU8701, which contains the *sigE* deletion, formed less than 10 heat-resistant spores per ml after incu-

FIG. 3. Southern blot analysis of *sigE* deletion. Chromosomal DNA from strains JH642 (lanes a and c) and EU8701 (lanes b and d) were cleaved with *EcoRI*, and the fragments were separated by electrophoresis in a 0.8% (wt/vol) agarose gel. The fragments were then transferred to nitrocellulose and hybridized with the radiolabeled 1.1-kb *PstI* fragment from pGSII11 (lanes a and b) and 0.3-kb *HincII-HindIII* fragment from pTK3 (lanes c and d). Shown is an autoradiograph of the nitrocellulose blot after hybridization. Each lane came from the same agarose gel. Fragment sizes in kilobases are shown in the margins.

TABLE 2. Complementation analysis of *spoIIIG* mutants

Strain	Relevant genotype ^a	No. of heat-resistant spores/ml
JH642	<i>trpC2 phe-1</i>	9×10^9
BS50	<i>spoIIIG41</i>	1×10^4
EU8745	BS50 SP β <i>sigE</i>	6×10^9
EU8746	BS50 SP β <i>gis</i>	1×10^4
EU8701	JH642 Δ <i>sigE::erm</i>	<10
EU8735	EU8701 SP β <i>sigE</i>	6×10^9
EU8736	EU8701 SP β <i>gis</i>	4×10^1
EU8725	JH642 Ω pTK15	4×10^5
EU8737	EU8725 SP β <i>sigE</i>	8×10^9
EU8738	EU8725 SP β <i>gis</i>	3×10^5
EU8719	JH642 Ω pTK9	2×10^5
EU8739	EU8719 SP β <i>sigE</i>	3×10^5
EU8740	EU8719 SP β <i>gis</i>	3×10^5
EU8723	JH642 Ω pTK13	3×10^5
EU8741	EU8723 SP β <i>sigE</i>	4×10^5
EU8742	EU8723 SP β <i>gis</i>	2×10^5

^a See Table 1, footnote a.

bation for 24 h in liquid sporulation medium, whereas the parental strain, JH642, formed 9×10^9 heat-resistant spores per ml under the same conditions (Table 2). The sporulation-defective phenotype of EU8701 was complemented to sporulation proficiency (Spo⁺) by lysogenization with the specialized transducing phage SP β *sigE* (EU8735; Table 2). It is likely that the SP β *sigE* prophage enabled EU8735 to sporulate by complementation of the *sigE* mutation and not by recombination, since loss of the prophage from EU8735 resulted in a Spo⁻ phenotype (data not shown) and lysogenization with an alternative specialized transducing phage (SP β *gis*) that contained the *sigE* structural gene in the opposite orientation to that in SP β *sigE* but was otherwise identical did not allow spore formation (EU8736; Table 2). It is likely that *sigE* is expressed in SP β *sigE* from a promoter located within the phage genome since *sigE* was not expressed when the *sigE* coding sequences were in the orientation found in SP β *gis*. Since deletion of *sigE* prevented sporulation and a specialized transducing phage containing only *sigE* complemented this defect, we concluded that *sigE* was essential for sporulation. Moreover, the deletion of *sigE* evidently did not have an adverse polar effect on the expression of an adjacent sporulation-essential gene.

Use of integrational plasmids to define the transcriptional unit. Nucleotide sequence analysis by Kobayashi and Anaguchi (11) revealed that the region between the *sigE* gene and the *PstI* site located 600 bp upstream from *sigE* does not contain a consensus-type promoter but does contain one large open reading frame (ORF) that may be translated in the same direction as *sigE* is. The exact size of this putative ORF is unknown because the nucleotide sequence of the region upstream from the *PstI* site was not determined. The termination codon (TAG) of this ORF is separated by only 13 bp from the start codon (ATG) of *sigE*; therefore, it is possible that *sigE* and this upstream ORF are part of the same transcriptional unit.

To define the transcriptional unit that includes *sigE*, we used integrational plasmids in a strategy similar to that first used by Piggot et al. (16) in their study of the organization of the polycistronic sporulation-essential operon *spoIIA*. The integrational plasmids encoded a chloramphenicol acetyltransferase that in *B. subtilis* resulted in a chloramphenicol-resistant phenotype. They did not replicate autonomously in *B. subtilis*, but because they each carried a fragment of DNA from the *spoIIIG* region, they generated stable chloramphen-

FIG. 4. Integrational plasmids. A partial restriction map of the *spoIIG* region of the *B. subtilis* chromosome is shown at the top. The open bar represents the *sigE* coding region, which is transcribed from left to right. The numbers are the distances in kilobases between restriction sites. Restriction enzyme cleavage sites indicated are as follows: RI, *EcoRI*; P, *PstI*; T, *TaqI*; RV, *EcoRV*. The fragment in each integrational plasmid that is homologous to the chromosome is indicated by the line under the restriction map. The phenotype that resulted from a Campbell-type integration of the plasmid is indicated as Spo⁺ (sporulation proficient) or Spo⁻ (sporulation deficient). The number of heat-resistant spores produced by each strain that contained an integrated plasmid is indicated. Strain JH642 produced 9×10^9 spores per ml under the same conditions.

icol-resistant *B. subtilis* transformants by single reciprocal recombinational events with the homologous region on the chromosome. This recombination resulted in a Campbell-type (16) insertion of the circular plasmids into the chromosome. If the region of homology on the plasmid comes from within a transcriptional unit, then this insertion should disrupt the transcriptional unit. However, if the region of homology contains at least one end of the transcriptional unit, then the Campbell-type integration should generate one complete functional transcriptional unit (see reference 16 for a more detailed explanation). We took advantage of this process to study the organization of the *spoIIG* operon by using a series of integrational plasmids to define the ends of the transcriptional unit.

Fifteen integrational plasmids were constructed as described in Materials and Methods by cloning different DNA fragments from the *spoIIG* region of the *B. subtilis* chromosome into pJH101 (Fig. 4 and 5). *B. subtilis* JH642 was transformed with each integrational plasmid, and chloramphenicol-resistant transformants were obtained. As expected, no chloramphenicol-resistant transformants were obtained when pJH101, the parental plasmid that did not contain a region homologous to the *B. subtilis* chromosome, was used to transform *B. subtilis*. Southern blot analysis (13) of the chromosomal DNA from each chloramphenicol-resistant strain was used to confirm that the plasmid had integrated as expected by a Campbell-type event into the *spoIIG* region of the chromosome. For this analysis, the DNA from each strain was cleaved with restriction enzyme, and fragments were separated by electrophoresis in agarose

gels and then transferred to nitrocellulose by ascending chromatography. The separated fragments were hybridized with several radiolabeled probes, including DNA fragments from the *spoIIG* region, to show that the fragments from the *spoIIG* region had been altered and with a pJH101 probe to identify the location of the integrated plasmid. In most instances the experiment was repeated after cleavage of the chromosomal DNA with a different restriction enzyme, and in each instance we found that the integrational plasmid had recombined with the homologous *spoIIG* region by a Campbell-type mechanism.

We expected that insertion of any integrational plasmid that prevented the synthesis of σ^E would result, like the deletion of the *sigE* gene in strain EU8701, in a sporulation-deficient phenotype. Each *B. subtilis* strain that contained a different integrational plasmid was tested for its ability to form endospores (Fig. 4 and 5). The region of homology in integrational plasmid pTK3 came entirely from within the *sigE* coding region; therefore, integration of this plasmid disrupted the *sigE* coding sequence. As expected, EU8713, which contained the integrated pTK3, produced unpigmented colonies on DSM plates, indicating that it did not sporulate efficiently. In liquid sporulation medium, EU8713 formed spores very inefficiently (2×10^5 spores per ml as compared with 9×10^9 spores per ml formed by the wild-type parental strain JH642). We were surprised to find that EU8713 appeared to sporulate somewhat more efficiently than did EU8701, which contained the *sigE* deletion. The EU8713 survivors of the heat test gave rise to unpigmented colonies that were resistant to chloramphenicol. Based on this observation, we concluded that they had a Spo⁻ phenotype and had not lost the integrated plasmid. It may be that homologous recombination leading to a temporary loss of the integrated plasmid occurred at a low frequency after the cells had stopped dividing, thereby allowing synthesis of σ^E . Reintegration of the plasmid may have occurred before it was lost from the dividing, germinated cells.

Integrational plasmid pTK4 contained a fragment that included the promoter-distal end of the *sigE* coding region and sequences that are downstream from this end of *sigE* (Fig. 4). Integration of this plasmid (strain EU8714) resulted in the generation of a complete *sigE* coding region. Strain EU8714 formed pigmented colonies that appeared to be Spo⁺ on DSM plates. In liquid sporulation medium its ability to form spores was slightly impaired (Fig. 4). This result is discussed below.

The integrational plasmids pTK1 and pTK2 contained the promoter-proximal end of the *sigE* coding sequence and 180 bp that are upstream from the start of the *sigE* coding sequence (Fig. 4). Strains that contained these integrated plasmids sporulated as inefficiently as did EU8713, in which the *sigE* coding region was disrupted (Fig. 4). Since the integration of pTK1 and pTK2 did not disrupt the *sigE* coding region, it seems likely that integration of these plasmids separated *sigE* from its promoter. Integration of plasmid pTK15 (Fig. 5), which contained sequences from within the *sigE* coding region to the *AhaIII* restriction endonuclease cleavage site located 700 bp upstream from the *sigE* coding sequence, also resulted in a Spo⁻ phenotype (Fig. 5). Because integration of pTK15 did not disrupt the *sigE* coding sequences, there are three possible explanations for the Spo⁻ phenotype. First, the insertion of pTK15 may have separated *sigE* from its promoter, which would have to be located upstream from the *AhaIII* cleavage site. This explanation assumes that the Spo⁻ phenotype is caused by

FIG. 5. Integrational plasmids. The figure is similar to Fig. 4 except that the *spoIIG* promoter region is expanded. The additional restriction sites indicated are *Aha*III (A) cleavage sites.

absence of *sigE* expression; therefore, lysogenization of this strain with a specialized transducing phage carrying only *sigE* would be expected to result in a Spo⁺ phenotype. Second, the insertion of pTK15 could have disrupted a second sporulation-essential gene, in which case a phage that carried only *sigE* would not be able to convert the strain to a Spo⁺ phenotype. Since EU8725, the strain with the pTK15 insertion, was complemented for sporulation by the introduction of the specialized transducing phage SPβ*sigE*, which carried the *sigE* gene (strain EU8737; Table 2), the second explanation was eliminated. A third explanation is that the Spo⁻ phenotype of EU8725 was due to the absence of *sigE* expression that was caused by the disruption of a gene that encodes a *trans*-acting factor which is essential for activation of the *sigE* promoter. This explanation is unlikely because the region in pTK15 that is homologous with the chromosome would have to have come entirely from within this essential gene. Therefore, this gene would have to overlap with *sigE* and extend more than 700 bp upstream from *sigE*. Moreover, the insertion of pTK15 did not disrupt an ORF (11). Therefore, the *trans*-acting factor could not be a protein. Finally, in experiments described below the *sigE* promoter was mapped independently to the region upstream from the *Aha*III site by using derivatives of a promoter probe plasmid. We conclude that the Spo⁻ phenotype of EU8725 was due to the absence of *sigE* expression, which probably occurred because the insertion had separated *sigE* from its promoter.

A second sporulation-essential gene in the *spoIIG* operon. The effects of the integrational plasmids described thus far indicate that the promoter for *sigE* is located at least 700 bp upstream from the *sigE* coding sequence. The results obtained with the additional integrational plasmids shown in Fig. 4 and 5 are consistent with the hypothesis that the promoter for *sigE* is located about 1 kb upstream from the *sigE* coding sequences within the 217-bp *Taq*I-*Aha*III fragment illustrated in Fig. 5. Independent evidence for the existence of the promoter in this region is described below.

If the promoter is located about 1 kb upstream from *sigE*, then it seemed possible that a second gene could be located in the region between the promoter and *sigE*. The integration of pTK9 (strain EU8719; Table 2), which contained the 500-bp *Pst*I DNA from this region, resulted in a Spo⁻ phenotype (Fig. 4). This strain was not complemented for sporulation by lysogenization with SPβ*sigE* (EU8739; Table 2). Introduction of this phage into the sporulation-defective strain BS50, which contains the *spoIIG41* allele of *sigE* allowed the cells to sporulate (EU8745; Table 2). It is likely that the prophage enabled this strain to sporulate by com-

plementation of the *sigE* mutation and not by recombination since lysogenization with the specialized transducing phage (SPβ*gis*, containing the *sigE* structural gene in the opposite orientation) did not complement it (EU8746; Table 2). Because the Spo⁻ phenotype resulting from insertion of pTK9 could not be complemented by the wild-type allele of *sigE* in SPβ*sigE*, we conclude that a second gene essential for sporulation is located between the promoter for *sigE* and the *sigE* coding sequence. In keeping with previous nomenclature for polycistronic sporulation operons, we refer to this gene as *spoIIGA*, whereas we refer to the promoter-distal gene of the operon as *sigE* because it encodes σ^E. Since the sporulation-deficient phenotype of strain EU8723, containing the integrated pTK13, also was not complemented for sporulation by the *sigE* transducing phage (EU8741; Table 2), it is likely that the coding region of *spoIIGA* is larger than 760 bp, extending from near *sigE* to a position upstream from the 160-bp *Aha*III DNA fragment (Fig. 5). The putative ORF in this region that was identified by Kobayashi and Anaguchi (11) may be the same as *spoIIGA*.

Analysis of promoter activity by using gene fusions. The integrational plasmid analysis led us to conclude that the promoter for the *sigE* gene must be located upstream from the *Aha*III cleavage site about 700 bp from *sigE* but downstream from the *Taq*I cleavage site (Fig. 4 and 5). To independently test for promoter activity, several DNA fragments were cloned into the promoter probe plasmid pLC1 as described in Materials and Methods. The inserted DNA fragments in these pLC1 derivatives were oriented such that the *sigE* promoter could direct transcription of a promoterless derivative of the *xylE* gene, which encodes CatO₂ase. A pLC1 derivative, pTK2200, which contains the 2,200-bp *Eco*RI DNA fragment from pTK7 (Fig. 4) was transformed into *B. subtilis*, in which it replicates autonomously. A transformant was incubated in liquid sporulation medium (DSM), and CatO₂ase activity was assayed at hourly intervals as described in Materials and Methods. CatO₂ase accumulated only after the end of exponential growth as the cells began to form endospores, accumulating to 3 mU/mg of protein by hour 2 of sporulation. *B. subtilis* that contained pLC4, the parental plasmid, did not produce CatO₂ase activity.

Smaller DNA fragments were cloned into pLC1 to determine the location of the promoter activity in the 2,200-bp *Eco*RI DNA fragment. The pLC1 derivative pTK800 contained the 800-bp *Aha*III-*Eco*RI DNA fragment from pTK15 (Fig. 5). Cells containing pTK800 did not produce CatO₂ase; therefore, the promoter activity in the 2,200-bp *Eco*RI fragment in pTK2200 must be located upstream from the *Aha*III

FIG. 6. Time course of *spoIIG*-*xylE* induction. Shown is the specific activity of CatO₂ase in strain JH642 containing either pTK480 (●), which contains the 480-bp *TaqI*-*PstI* DNA fragment from the *spoIIG* promoter region, or pTK500 (○), which contains 500 bp from the promoter-proximal end of the 1.1-kb *PstI* DNA fragment that encodes *sigE*. The cells were grown in DSM, and growth was measured by the OD₅₅₀ (▲).

site. We also examined the promoter activity of the DNA between the *HindIII* site within *sigE* (codon 109) and the *PstI* site located 160 bp upstream from *sigE*. This DNA fragment in pTK500 also did not promote production of CatO₂ase (Fig. 6). The 480-bp *TaqI*-*PstI* DNA fragment from pTK11 was cloned into pLC1. This recombinant plasmid, pTK480, produced CatO₂ase during the first 2 h of sporulation slightly more rapidly than did pTK2200, which contained the 2,200-bp *EcoRI* DNA fragment (Fig. 6). We conclude that in this assay, the 480-bp *TaqI*-*PstI* DNA fragment had promoter activity which, in the chromosome, would direct transcription toward *spoIIGA* and *sigE* and that no promoter activity was located between this promoter and *sigE*. Since the results with the integrational plasmids were consistent with the hypothesis that the promoter for *sigE* and *spoIIGA* is located within the 480-bp *TaqI*-*PstI* DNA fragment, it is likely that the expression of *xylE* that we observed was due to the same promoter, henceforth referred to as the *spoIIG* promoter.

A smaller region within the 480-bp *TaqI*-*PstI* fragment, the 217-bp *TaqI*-*AhaIII* DNA fragment (Fig. 5), also was cloned into a pLC1 derivative. When this plasmid (pLC217) was transformed into *B. subtilis* and CatO₂ase activity was monitored, we found that CatO₂ase accumulated at the same rate as when the entire *TaqI*-*PstI* fragment was present. Based on this observation, we concluded that the *spoIIG* promoter was located within the 217-bp *TaqI*-*AhaIII* DNA fragment.

Effects of mutations on expression from the *spoIIG* promoter. We fused the 480-bp *TaqI*-*PstI* DNA fragment that contains the *spoIIG* promoter to a promoterless derivative of *lacZ* from *E. coli* and put this construction into the specialized transducing phage SPβ to facilitate transfer to different strains and to eliminate potential problems associated with the use of multicopy plasmids (27) (see Materials and Methods; Fig. 2). This strategy has been used previously by Igo and Losick (9), and they found that β-galactosidase was produced above background levels only when a promoter was added to this type of *lacZ* fusion in SPβ. A sporulation-proficient strain that carried prophage SPβIIG-*lacZ* (EU8743), which contained the *spoIIG*-*lacZ* fusion was induced to sporulate by the addition of decoyinine. The

accumulation of β-galactosidase began immediately after the initiation of sporulation and increased to about 7 Miller units by hour 3 of sporulation (Fig. 7A). The parental strain JH642, which did not contain this prophage, produced about 0.5 to 1.0 Miller unit of β-galactosidase. When SPβIIG-*lacZ* lysogens and the sporulation-deficient lysogens described below were grown in liquid sporulation medium (DSM) in which sporulation was induced by nutrient depletion, the rate of accumulation of β-galactosidase by each strain was the same as when each strain was treated with decoyinine. The pattern of accumulation of β-galactosidase after the initiation of sporulation was similar to the pattern of accumulation of CatO₂ase seen when expression of *xylE* was directed by the *spoIIG* promoter (cf. Fig. 6 and 7).

Mutants that were blocked at early stages of spore formation were made lysogenic for SPβIIG-*lacZ*. Mutations at *spo0A*, *spo0B*, *spo0H*, and *spo0F* all caused a dramatic decrease in the rate of β-galactosidase accumulation (Fig. 7). Mutations at *spo0J* and *sigB*, the structural gene for σ^B (formerly σ³⁷), did not affect the pattern of β-galactosidase accumulation (Fig. 7). A deletion at *sigE* or a nonsense mutation at *spoIIC* caused the rate of accumulation of β-galactosidase and the amount produced to increase (Fig. 7). A mutation at *abrB* can suppress the inefficient transcription from the *spoVG* promoter caused by mutations in *spo0A*. We did not observe β-galactosidase synthesis in the SPβIIG-*lacZ* lysogen that had mutant alleles at both the *abrB* and *spo0A* loci (EU8728) (Fig. 7). We did observe wild-type levels of β-galactosidase synthesis directed by the *spoVG* promoter in an SPβ::*spoVG*-*lacZ* lysogen of this *spo0A* *abrB* mutant (data not shown). These results are discussed below.

Nucleotide sequence of the region that includes the *spoIIG* promoter. The nucleotide sequence of the 217-bp *TaqI*-*AhaIII* fragment that contains the *spoIIG* promoter, determined as described in Materials and Methods, includes a sequence that is similar to promoters that are used by Eo⁴³ (Fig. 8). We do not make any definitive conclusion about this observation because we do not know the exact start point of transcription or the effects of mutations in this region on expression of the *sigE* gene.

Analysis of the RNA transcripts of the *spoIIG* operon. For Northern blot analysis, we prepared RNA from vegetative cells in the exponential growth phase and from sporulating cells, as described in Materials and Methods. These RNAs were hybridized with radiolabeled probes derived from various portions of the *spoIIG* operon and from the region adjacent to the promoter-proximal end of the operon (Fig. 9). We detected two transcripts in the RNA from sporulating cells that were absent from the RNA from vegetative cells. A 950-base sporulation-specific transcript was detected with the probe from the *sigE* coding sequence (Fig. 9, lanes m and o). A second sporulation-specific transcript (1.2 kb) was detected with the 480-bp *TaqI*-*PstI* DNA fragment and the 500-bp *PstI* DNA fragment (Fig. 9, lanes g and i, respectively). In some experiments, we observed slight hybridization between this transcript and the 1.1-kb *PstI* DNA fragment. No sporulation-specific transcript was identified with the 1-kb *EcoRI*-*TaqI* DNA fragment that came from the region adjacent to the promoter-proximal end of the *spoIIG* operon. Therefore, the end of the 1.2-kb sporulation-specific transcript must lie within the 480-bp *TaqI*-*PstI* fragment, which contains the *spoIIG* promoter. Although no sporulation-specific transcript was detected with the 1-kb *EcoRI*-*TaqI* probe, a 2.2-kb transcript from vegetative cells hybridized with this probe (Fig. 9, lane b). With all the probes, we

FIG. 7. Effects of mutations on *spoIIIG-lacZ* induction. Strains that contained the SP β ::*spoIIIG-lacZ* prophage were induced to sporulate by the addition of decoyinine (time zero), and β -galactosidase was monitored at hourly intervals. (A) Symbols: $\circ$, EU8743 (Spo⁺); $\square$, EU8728 (*spo0A abrB*); $\blacktriangle$, EU8727 (*spo0A*). (B) Symbols: $\bullet$, EU8744 (Δ *sigE*); $\blacktriangle$, EU8731 (*spo0J*); $\circ$, EU8729 (*spo0B*); $\square$, EU8730 (*spo0F*). (C) Symbols: $\circ$, EU8726 (*spoIIAC*); $\blacktriangle$, EU8733 (*spo0H*); $\square$, EU8732 (*spo0H81*); $\blacksquare$, EU8734 (*sigB*).

found two larger transcripts (about 1.5 and 2.6 kb) from both vegetative and sporulating cells. These were probably the result of nonspecific hybridization to rRNA, since they also hybridized to RNA copies of each strand of the 480-bp *TaqI-PstI* DNA fragment (data not shown).

DISCUSSION

The evidence that is presented here supports a model in which the *spoIIIG* operon consists of two sporulation-essential genes: a promoter-proximal gene, *spoIIGA*, and a promoter-distal gene, *sigE*, which is the structural gene for σ^E . The deletion of *sigE* produced a sporulation-deficient phenotype. This strain was complemented for sporulation by a prophage expressing *sigE* (SP β *sigE*); therefore, *sigE* expression is essential for sporulation. The integration of pTK15, which did not disrupt *sigE*, also prevented sporulation by preventing *sigE* expression, since the sporulation-defective strain was also complemented by SP β *sigE*. pTK15 contains the region extending 700 bp from *sigE* toward the promoter. The insertion of pTK15 must have separated *sigE* from its promoter; therefore, the promoter is at least 700 bp upstream from *sigE*. The promoter must be downstream

from the *TaqI* site because insertion of plasmids that contained sequences upstream from this region did not prevent sporulation (i.e., did not prevent *sigE* expression) (e.g., pTK5, pTK7, and pTK11). This *TaqI-PstI* region upstream from *sigE* was shown to have promoter activity by fusing it to promoterless derivatives of *xylE* on a plasmid and to *lacZ* on a transducing phage; promoter activity was not detected between this region and *sigE*.

A second sporulation-essential gene is located between *sigE* and its promoter, since the integration of pTK9 in this region resulted in a Spo⁻ phenotype that was not complemented by SP β *sigE*. If this second sporulation-essential gene, *spoIIGA*, is transcribed in the same direction as is *sigE*, then the promoter for *spoIIGA* is the same as or overlaps with the *sigE* promoter. It is likely that *spoIIGA* is transcribed in the same direction as *sigE* is because disruption of *spoIIGA* with pTK9 disrupted only one ORF and this ORF is translated in the same direction as is *sigE* (11). The function of this promoter-proximal gene, *spoIIGA*, is unknown, but it may be involved in the processing of P³¹, the precursor of σ^E , since mutations in this gene appear to cause accumulation of P³¹ (W. G. Haldenwang, personal communication).

It is unlikely that transcription from the *spoIIIG* promoter is necessary for expression of a sporulation-essential gene located downstream from the *sigE* gene. Insertion of pTK15 in strain EU8725 caused a polar mutation that prevented expression of *sigE*; therefore, this insertion would also be expected to prevent transcription of a gene located downstream. Since the Spo⁻ phenotype of the pTK15 insertion could be overcome by expression of *sigE* from a prophage, expression of an additional sporulation-essential gene downstream is not prevented by the insertion. The slightly defective sporulation phenotype associated with the insertion of pTK4 deserves comment. As described above, we do not think that this insertion blocks transcription to a sporulation gene downstream. We speculate that the insertion of plasmid sequences upstream from a promoter for a gene located adjacent to the promoter-distal end of the *spoIIIG* operon may have adverse consequences on full utilization of the promoter.

The appearance of σ^E is controlled at two levels: mRNA accumulates only after the initiation of sporulation, and the

```

TaqI                                     50
TCGACAAATT AAGCAGATTT CCCTGAAAAA TTGTATTTTC CTCTCAACAT

-35          -10          100
TAATTGACAG ACTTCCAC AGAGCTTGCT TTATACTTAT GAAGCAAGAA

GGGGAACAGC GTGAGGCAAG AAAGAAAGGA GTCAGATGTG AAAATCTATT

200
TAGATGTCAT TTGGCTGTTA AACTTTTGTG TTGATGCACT TTGCTTTTG

AhaIII
CTCACGGCAT TTATTTTAAA

```

FIG. 8. Nucleotide sequence of the nontranscribed strand of the *spoIIIG* promoter region. Transcription into the *spoIIIG* operon occurs from left to right. The lines above the sequence indicate sequences that are similar to the sequences at the -10 and -35 regions of promoters that are used by E σ^{43} . The underlined sequence identifies a region of dyad symmetry.

FIG. 9. Northern blot analysis of RNA transcripts from the *spoIIG* region. RNA was isolated from *B. subtilis* JH642 (Spo^+) during mid-exponential growth (vegetative cells) and during hour 1 of sporulation. After denaturation, 20- μ g samples of each RNA preparation were loaded into alternate wells of a formaldehyde-agarose slab gel and subjected to electrophoresis. After electrophoresis, the RNA was transferred to nitrocellulose, and the nitrocellulose sheet was cut into four strips, each of which contained a lane of RNA from vegetative cells (V) and a lane of RNA from sporulating cells (S). These nitrocellulose strips were hybridized with the radiolabeled DNA fragments shown above the autoradiographs: the 1-kb *EcoRI*-*TaqI* fragment from pTK6 (lanes b, c, d, and e); the 480-bp *TaqI*-*PstI* fragment from pTK11 (lanes f and g); the 500-bp *PstI* fragment from pTK9 (lanes h and i); and the 1.1-kb *PstI* fragment from pGSIIG11 (lanes l, m, n, and o). Lanes d and e are longer exposures of lanes b and c, respectively, and lanes n and o are longer exposures of lanes l and m, respectively. Relative molecular weight standards (the 1-kb DNA ladder; Bethesda Research) are shown in lanes a and j. The arrowheads indicate the positions of transcripts that were found in the RNA from sporulating cells but not from vegetative cells. Shown above the autoradiographs is a partial restriction map of the *spoIIG* region. The cleavage sites for *EcoRI* (R), *TaqI* (T), and *PstI* (P) are indicated. The numbers indicate the distances in kilobases between cleavage sites, and the open bar indicates the *sigE* coding region. The wavy arrows indicate the positions to which the two sporulation-specific transcripts map.

product of *sigE* must be posttranslationally processed to active σ^E (23; W. G. Haldenwang, personal communication). The accumulation of mRNA appears to reflect increased promoter activity at the beginning of sporulation. This conclusion is based on our finding that expression of *xylE* and *lacZ* from the *spoIIG* promoter increased at that time. We can now begin to construct some models about the regulation of *spoIIG* promoter activity. Since the promoter was active in *spo0J* and *sigB* mutants, we can conclude that the product of *spo0J* and the σ^B -containing RNA polymerase are not needed for utilization of the *spoIIG* promoter. A mutation at *spoIIG* increased utilization of the *spoIIG* promoter, but this apparent autoregulatory activity is probably indirect, since a mutation in another gene that blocks sporulation at stage II, *spoIIAC1*, also caused an increased rate of expression from the *spoIIG* promoter. It may be that as the cells develop past stage II, a product is produced that prevents use of the *spoIIG* promoter, and mutations that block development at stage II and therefore block production of the negative-acting product cause increased use of the *spoIIG* promoter. An alternative explanation (suggested by a reviewer of this manuscript) is that the absence of competing sigma factors, encoded by *sigE* and *spoIIAC*, favors the expression of the *spoIIG* promoter through another sigma factor.

Mutations in the *spo0* genes blocked the initiation of spore formation. The products of several *spo0* genes are directly or

indirectly needed for *spoIIG* promoter activity, since the promoter was less active in *spo0A*, *spo0B*, *spo0F*, and *spo0H* mutants. The *spoVG* promoter, like the *spoIIG* promoter, is activated at the beginning of sporulation, and activation of both promoters requires the products of the same set of *spo0* genes (28; this study). A mutation at the *abrB* locus partially suppresses the phenotype produced by a mutation at *spo0A*, restoring transcription from the *spoVG* promoter but not allowing the cells to form spores (29). We found that this mutation at *abrB* did not restore *spoIIG* promoter activity in a *spo0A* mutant. The absence of *spoIIG* promoter activity in a *spo0A abrB* mutant may explain why this mutant cannot form spores and demonstrates that the role of *spo0A* in *spoIIG* transcription is at least slightly different from its role in *spoVG* transcription. Losick et al. (12) have suggested that the role of the *spo0A* gene product in *spoVG* transcription may be to inactivate the *abrB* product, which may be a negative regulator of *spoVG* transcription. For transcription of *spoIIG*, the *spo0A* gene product may have to inactivate a different negative regulator of the *spoIIG* promoter. In any event, it is apparent that these two promoters (*spoIIG* and *spoVG*) that are activated early during sporulation are regulated differently.

Northern blot analysis showed that two transcripts from the *spoIIG* operon accumulated in sporulating cells. One transcript was homologous with the region between the *spoIIG* promoter and the start of the *sigE* gene, whereas the

other was from the *sigE* region. Because of the effects of the integrational plasmids and the observation that the region encoding the 5' end of the *sigE* transcript did not have promoter activity in the *xylE* fusion plasmid, we suggest that these two transcripts are rapidly processed from a larger precursor that is transcribed from the *spoIIIG* promoter. Several mRNAs have been shown to be processed endonucleolytically in *E. coli* and *B. subtilis* (10). Our model is similar to the observation by Burton et al. (2) that the polycistronic mRNA that encodes *rpoD*, the structural gene for the primary RNA polymerase sigma factor in *E. coli*, is endonucleolytically cleaved at the junction of *rpoD* and the adjacent promoter-proximal gene, *dnaG*. A region of dyad symmetry that includes the ribosome-binding site of *sigE* may be the signal recognized by the processing enzyme.

Transcription from the *spoIIIG* promoter appears to be activated after the initiation of sporulation, raising two interesting questions. How is promoter activity regulated, and which of the several forms of RNA polymerase in *B. subtilis* uses this promoter? Because the promoter activity of the 480-bp *TaqI*-*PstI* DNA fragment was not diminished by removal of all but the 217 bp located between the *TaqI* and *AhaIII* sites, we conclude that the promoter sequences are located in this region. Recently, we have found that a sequence in this fragment can be used by the primary form of RNA polymerase found in vegetative cells ($E\sigma^{43}$) in an in vitro transcription assay (unpublished data), but we do not know if $E\sigma^{43}$ utilizes this promoter in vivo. We speculate that if $E\sigma^{43}$ transcribes the *spoIIIG* operon, then the sequence of dyad symmetry found overlapping the $E\sigma^{43}$ promoter (Fig. 8) may be involved in the binding of a regulatory protein.

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