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Spo0A Controls the σ^A-Dependent Activation of *Bacillus subtilis* Sporulation-Specific Transcription Unit *spoIIE*

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The *spoIIE* operon is a developmentally regulated transcription unit activated in the second hour of sporulation in *Bacillus subtilis*. Its promoter has an unusual structure, containing sequences which conform perfectly to the consensus for vegetative promoters recognized by σ^A-associated RNA polymerase ($E\sigma^A$), but with a spacing of 21 bp between the apparent −10 and −35 elements instead of the 17- or 18-bp spacing typical of promoters utilized by $E\sigma^A$. Mutations introduced into the apparent −10 element affected transcription in a manner consistent with its functioning as a polymerase recognition sequence. The deleterious effect of one −10 mutation was also suppressed in an allele-specific manner by a mutation in *sigA* known to suppress analogous −10 mutations in conventional vegetative promoters recognized by $E\sigma^A$. Similar suppression experiments failed to provide evidence for a direct interaction between $E\sigma^A$ and the "−35-like" element, however, and DNase I protection experiments suggested instead that the Spo0A protein binds to a site overlapping this −35-like hexamer. Moreover, the effects of mutations within the −35-like hexamer on the binding of Spo0A in vitro paralleled their effects on transcription in vivo. We suggest that *spoIIE* belongs to a class of early-intermediate sporulation genes whose transcription by $E\sigma^A$ is activated by the Spo0A protein.

Under conditions of nutrient limitation, *Bacillus subtilis* bacteria initiate a differentiation process which culminates in the formation of dormant endospores. This complex series of biochemical and morphological changes, which probably involves over 100 gene products, requires the temporally regulated activation of many transcription units (20). Changes in gene expression are driven, in part, by the sequential appearance of new sigma factors, which bind to core RNA polymerase (E) and confer on the holoenzyme ($E\sigma$) the capacity to recognize new classes of sporulation-specific promoters (40). Known examples include σ^H, which participates in the activation of early genes, σ^E and σ^F, which regulate early-intermediate genes, and σ^G and σ^K, which control the compartment-specific expression of gene sets activated at later times in the developing cell.

Each class of promoters has a characteristic and distinct polymerase consensus sequence, typically centered about 10 and 35 bp upstream of the transcription start site (27), an observation which formed the basis for the model that σ directs RNA polymerase to its cognate promoters by making sequence-specific contacts in these regions (19). The most convincing evidence for this model comes from experiments in which single amino acid changes in the σ protein were found to alter its promoter recognition specificity (9, 37, 42, 49). These experiments suggest a direct interaction of σ with DNA and have identified regions of the σ protein that are likely to contact the −10 and −35 regions of promoters.

The *spoIIE* operon is a developmentally regulated, sporulation-specific transcription unit activated in the second hour of sporulation (11). Its promoter region does not contain sequences that conform to the consensus for any of the known sporulation-specific σ factors. Instead, it contains sequences that conform perfectly to the consensus sequence for vegetative promoters recognized by σ^A-associated RNA polymerase ($E\sigma^A$), but with a spacing of 21 bp between the −10 and −35-like elements, rather than the preferred 17- to 18-bp spacing typical of vegetative promoters. Interestingly, another sporulation-specific operon, *spoIIG*, which is activated at the same time as *spoIIE*, has a similar promoter structure (13).

Previous work has shown that the sequences resembling σ^A recognition hexamers in the *spoIIG* promoter are important for transcriptional activity (13, 35), and allele-specific suppression experiments have provided evidence that $E\sigma^A$ recognizes the *spoIIG* promoter in vivo (16). In the present work, we found similar results for the *spoIIE* promoter. These findings seemed to reinforce the conclusion that $E\sigma^A$, the major vegetative form of polymerase, could somehow utilize promoters with inappropriately spaced recognition hexamers to activate transcription during sporulation. However, we also found genetic evidence and footprinting data suggesting that the sequence resembling a σ^A-like −35 recognition hexamer in the *spoIIE* promoter may actually function as a binding site for the Spo0A protein, a transcription factor known to control other gene activation events during the initiation of sporulation (41). Similar results for the *spoIIG* promoter are presented elsewhere (35). We propose that *spoIIE* and *spoIIG* are members of a regulon of $E\sigma^A$-dependent sporulation-specific transcription units activated by Spo0A. Furthermore, although the *spoIIE* and *spoIIG* promoters are transcribed by $E\sigma^A$, we suggest that the sequence resembling a σ^A −35 recognition hexamer in these promoters is not directly involved in interactions with RNA polymerase. Rather, it is, by coincidence, similar to sequences that bind Spo0A. Therefore we propose that the binding of Spo0A to these promoters compensates for the absence of a functional −35 element.

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TABLE 1. Bacterial strains used in this work

Strain	Relevant genotype or derivation	Source or reference
E. coli		
MM294	*endA thiA hsdR17 supE44*	2
DH5αF′	F′ ϕ80d*lacZ* ΔM15 Δ(*lacZYA-argF*) *endA1 recA1 supE44 thi-1 relA1 hsdR17* (r^-m^+) *gyrA* λ^-	Bethesda Research Labs
BW313	Hfr[*lysA*(*61-62*)] *dut1 ung1 thi-1 relA1*	17
B. subtilis		
BD170	*trpC2 thrA5*	D. Dubnau
EU9001	*glyB133 chr*::Tn*917*ΩHU160::P_{spac}*sigA*(wt)	16
EU9002	*glyB133 chr*::Tn*917*ΩHU160::P_{spac}*sigA*(QR196)	16
EU9004	*glyB133 chr*::Tn*917*ΩHU160::P_{spac}*sigA*(RH347)	15
GV57	*trpC2 thrA5*; lysogen SPβ*c2*Δ*2*::Tn*917*pSK10Δ6::pGV49	11
JH646	*pheA1 trpC2 spo0A12*	J. Hoch
KS160	*glyB133 chr*::Tn*917*ΩHU160	33
PY231	prototroph; pTV21Δ2	47
PY390	*spoIIJ*::Tn*917*ΩHU19	33
RS1729	*metC2 lys1*	T. Leighton
RS3060	*spoIIF96 lys1*	T. Leighton
ZB307	lysogen SPβ*c2*Δ*2*::Tn*917*::pSK10Δ6	50
ZB516	*trpC2 pheA1 spo0B136 abrB*::Tn*917*	P. Zuber
PY797	*trpC2 thrA5 abrB*::Tn*917*; transformation of BD170 with DNA from ZB516 selecting for Em^r	This work
PY798	*trpC2 spo0A12 abrB*::Tn*917*; transformation of PY797 with DNA from JH646 selecting for Thr^+, scoring for Spo^-	This work
KY82	*glyB133 chr*::Tn*917*ΩHU160::P_{spac}*sigA*(QR196); lysogen SPβ*c2*Δ*2*::Tn*917*pSK10Δ6::*spoIIE*(−14G)-*lacZ*	This work
KY83	*glyB133 chr*::Tn*917*ΩHU160::P_{spac}*sigA*(QR196); lysogen SPβc2Δ2::Tn*917*pSK10Δ6::*spoIIE*(−14C)-*lacZ*	This work
KY84	*glyB133 chr*::Tn*917*ΩHU160::P_{spac}*sigA*(QR196); lysogen SPβ*c2*Δ*2*::Tn*917*pSK10Δ6::*spoIIE*(wt)-*lacZ*	This work
KY87	*glyB133 chr*::Tn*917*ΩHU160::P_{spac}*sigA*(QR196); lysogen SPβ*c2*Δ*2*::Tn*917*pSK10Δ6::*spoIIE*(−9G)-*lacZ*	This work
KY74	*glyB133 chr*::Tn*917*ΩHU160::P_{spac}*sigA*(wt); lysogen SPβ*c2*Δ*2*::Tn*917*pSK10Δ6::*spoIIE*(−14G)-*lacZ*	This work
KY75	*glyB133 chr*::Tn*917*ΩHU160::P_{spac}*sigA*(wt); lysogen SPβ*c2*Δ*2*::Tn*917*pSK10Δ6::*spoIIE*(−14C)-*lacZ*	This work
KY76	*glyB133 chr*::Tn*917*ΩHU160::P_{spac}*sigA*(wt); lysogen SPβ*c2*Δ*2*::Tn*917*pSK10Δ6::*spoIIE*(wt)-*lacZ*	This work
KY85	*glyB133 chr*::Tn*917*ΩHU160::P_{spac}*sigA*(wt); lysogen SPβ*c2*Δ*2*::Tn*917*pSK10Δ6::*spoIIE*(−9G)-*lacZ*	This work
KY288	*glyB133 chr*::Tn*917*ΩHU160::P_{spac}*sigA*(RH347); lysogen SPβ*c2*Δ*2*::Tn*917*pSK10Δ6::*spoIIE*(−14G)-*lacZ*	This work
KY289	*glyB133 chr*::Tn*917*ΩHU160::P_{spac}*sigA*(RH347); lysogen SPβ*c2*Δ*2*::Tn*917*pSK10Δ6::*spoIIE*(−14C)-*lacZ*	This work
KY290	*glyB133 chr*::Tn*917*ΩHU160::P_{spac}*sigA*(RH347); lysogen SPβ*c2*Δ*2*::Tn*917*pSK10Δ6::*spoIIE*(wt)-*lacZ*	This work
KY291	*glyB133 chr*::Tn*917*ΩHU160::P_{spac}*sigA*(RH347); lysogen SPβ*c2*Δ*2*::Tn*917*pSK10Δ6::*spoIIE*(−39C)-*lacZ*	This work
KY292	*glyB133 chr*::Tn*917*ΩHU160::P_{spac}*sigA*(RH347); lysogen SPβ*c2*Δ*2*::Tn*917*pSK10Δ6::*spoIIE*(−39A)-*lacZ*	This work
KY21	*trpC2 thrA5 chr*::pGV49; transformation of BD170 with pGV49 selecting for Cm^r	This work
KY20	*trpC2 thrA5 chr*::pPP81; transformation of BD170 with pPP81 selecting for Cm^r	This work
KY255	*trpC2 thrA5 chr*::pZΔB.IIGwt; transformation of BD170 with pZΔB.IIGwt selecting for Cm^r	This work
KY269	*spo0A12 abrB*::Tn*917 chr*::pGV49; transformation of PY798 to Cm^r with DNA from KY21	This work
KY265	*trpC2 thrA5 abrB*::Tn*917 chr*::pGV49; transformation of PY797 to Cm^r with DNA from KY21	This work
KY261	*trpC2 spo0A12 chr*::pGV49; transformation of KY21 to Thr^+ with DNA from JH646, scoring for Spo^-	This work
KY277	*spoIIJ*::Tn*917*ΩHU19 *chr*::pGV49; transformation KY21 with chromosomal DNA from PY390 selecting for Em^r	This work
KY281	*metC2 lys1 chr*::pGV49; transformation of RS1729 to Cm^r with DNA from KY21	This work
KY282	*lys1 spoIIF96 chr*::pGV49; transformation of RS1729 to Cm^r with DNA from KY21	This work

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains used in this work are described in Table 1; plasmids and phages are described in Table 2.

Culture media and genetic techniques. Culturing of *B. subtilis* strains and selection for chloramphenicol resistance (Cm^r) and erythromycin resistance (Em^r) were performed as previously described (46). Phleomycin was used at a concentration of 0.5 μg/ml in Luria-Bertani (LB) plates buffered to pH 7.5 with 50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES; Sigma Chemical Co.) to select for phleomycin resistance (Pm^r) in *B. subtilis*. Phleomycin was obtained either from CAYLA S.A.R.L. or as a gift from Bristol Meyers Co. Competent cells of *B. subtilis* strains were prepared and transformed as described by Dubnau and Davidoff-Abelson (8). Techniques for the use of *B. subtilis* phage SPβ were as described by Rosenthal et al. (32). *Escherichia coli* strains and M13 derivatives were cultured or propagated as described by Messing (23). When appropriate, ampicillin was added to a concentration of 50 μg/ml. Transformation and transfection of *E. coli* strains were carried out as described by Lederberg and Cohen (18).

In vitro manipulation of DNA. Chromosomal DNA was isolated from *B. subtilis* strains as described previously (11). Preparative isolation of plasmid DNA was carried out by the alkaline lysis procedure of Birnboim and Doly (3), and the DNA was purified on CsCl gradients as described by Maniatis et al. (22). The method of Ish-Horowicz and Burke (12) was used for small-scale preparation of plasmid DNA. Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, and the Klenow fragment of DNA polymerase were purchased from Bethesda Research Laboratories or New England BioLabs and were used as recommended by the supplier. Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim Biochemicals. Except when noted, DNA sequencing was carried out by the dideoxy-chain termination method (34) on single-stranded DNA by using the Sequenase kit from United States Biochemical

TABLE 2. Plasmids and phages used in this work

Plasmid or phage	Relevant characteristics or derivation	Source or reference
pSCPS22	Contains *sigA*; similar to pSCPS2 (31), except that the *Eco*RI fragment containing *sigA* is in the opposite orientation (Fig. 1)	C. Price
pTV21Δ2	Contains left and right arms of Tn*917* interrupted by a ColE1-derived cloning vector carrying a *cat* gene selectable in *B. subtilis*	47
pAG58-*ble*-1	ColE1 derivative containing a P_{spac} expression cassette and a *ble* gene selectable in *B. subtilis*	48
pGV34	ColE1 derivative used for constructing *lacZ* fusions; carries a *cat* gene selectable in *B. subtilis*	11
pGV49	Derived from pGV34; carries a *spoIIE-lacZ* transcriptional fusion	11
pPP81	ColE1-derived vector; carries *spoIIA-lacZ* transcriptional fusion; *cat* gene	29
pZΔBIIGwt	Derived from GV34; carries a *spoIIG-lacZ* transcriptional fusion	35
M13mp19*cat*-32	Contains a 253-bp *Rsa*I-*Hin*dIII fragment including the entire *spoIIE* promoter region	11
M13mp18*sigA*	Contains a 1.7-kb *Sph*I-*Eco*RI fragment containing *sigA* inserted into the polylinker site of M13mp18	This work
M13mp18*sigA*(RH347)	Derived by site-directed mutagenesis from M13mp18*sigA* (Fig. 1)	This work
pSPIGMA	Contains a 1.7-kb *Hin*dIII fragment from M13mp18*sigA* inserted into pAG58-*ble* to place *sigA* under control of P_{spac} (Fig. 1)	This work
pSPIGMA(RH347)	Same as pSPIGMA, but contains mutant *sigA*	This work
pTV21-SPIGMA	P_{spac}-*sigA* cassette inserted between the Tn*917* arms in pTV21Δ2 (Fig. 1)	This work
M13mp18*cat*-8	274-bp *Eco*RI-*Hin*dIII fragment from pGV49 containing the *spoIIE* promoter subcloned into the polylinker of M13mp18-*cat* (11)	This work
M13mp18-9	640-bp *Eco*RI-*Bgl*I fragment from pGV49 including the *spoIIE* promoter and a portion of *lacZ* subcloned into the *Eco*RI-*Bgl*I backbone of M13mp18 (23)	This work

Corp. In addition to the Universal M13 primer, the following synthetic primers were used for DNA sequencing: 5′-GTCC CGCCATTGGCCCG-3′, which anneals to sequences within *spoIIE*; 5′-GGATGTGCTGCAAGGCG-3′, which anneals to sequences within *lacZ*; and 5′-CGTTTACTTCTGCTA GG-3′, which anneals to sequences near the C terminus of *sigA* (*rpoD*).

Mutagenesis of the *spoIIE* regulatory region. Site-directed mutagenesis of the *spoIIE* regulatory region was carried out essentially as described by Kunkel et al. (17), by using the mutagenic oligonucleotides listed in Table 3. Virion DNA of phage M13mp19*cat*-32 (Table 2) (11) was used as the template for second-strand synthesis. This construction contains a 253-bp segment of the *spoIIE* promoter region, which includes all sequences required for normal transcriptional regulation (−178 to +77, with respect to the transcription start site).

TABLE 3. Oligonucleotides used for site-directed mutagenesis

Mutation	Sequence (5′ to 3′)[a]
spoIIE −41T to G	GTTACCTTCTTGTGACAAAATCC
spoIIE −39G to C	ACCTTCTTTTCACAAAATCCTATC
spoIIE −13A to G	GTGCTTTCGCTGTAATGACAGGC
spoIIE −11A to G	GCTTTCGCTATGATGACAGGCAAC
spoIIE −9T to G	CTTTCGCTATAAGGACAGGCAACG
spoIIE −40T to C	TACCTTCTTTCGACAAAATCC
spoIIE −39G to A	ACCTTCTTTTAACAAAATCCTATC
spoIIE −45 TCT to GGA	TTGTTACCTGGATTGACAAAATCC
spoIIE −63 TCT to GGA	CGTCGAAGATTGGATTGGTATTGT
spoIIE −105 TCT to GGA	TTCCGGAAATGGATTTCATAAACG
spoIIE −22G to C	ATCCTATCTGTCCTTTCGCTATA
spoIIE −21C to A	TCCTATCTGTGATTTCGCTATAA
spoIIE −20T to G	CCTATCTGTGCGTTCGCTATAATG
spoIIE −19T to G	CCTATCTGTGCTGTCGCTATAATG
spoIIE −44C to G	GTTACCTTGTTTTGACA
spoIIE −62C to G	GAAGATTTGTTTGGTATT
spoIIE −14T to G	GCTTTCGCGATAATGACAGG
spoIIE −14T to C	GCTTTCGCCATAATGACAGG
sigA RH347	CTTCGATTTGATGAATACGCTCTC

[a] Bold type indicates the mutational substitution(s) in each oligonucleotide.

Evaluation of the effects of site-directed mutations. To construct an SPβ phage-borne transcriptional *lacZ* fusion to each mutant promoter, a 274-bp *Eco*RI-*Hin*dIII fragment, containing the *spoIIE* regulatory region, was subcloned from replicative-form DNA of the appropriate M13 clone into the *Eco*RI-*Hin*dIII backbone of pGV-34 (Table 2) (11). The resulting transcriptional *lacZ* fusions were integrated into SPβ prophage sequences as described by Zuber and Losick (50). The phage-borne transcriptional fusions were then introduced by transduction into *B. subtilis* BD170 (wild type) or mutant strains, selecting for Em^r.

β-Galactosidase assays. Cultures were grown for assay as previously described (11). Samples were assayed for activity by the fluorometric method of Youngman (46) or by the colorimetric method of Miller (24).

Construction of merodiploids with mutant *sigA* alleles. The 1.7-kb *Eco*RI-*Sph*I fragment from pCPS22 (Table 2), which contains the promoterless *sigA* (*rpoD*) coding sequence, was subcloned into the *Eco*RI-*Sph*I backbone of M13mp18. The resulting phage, M13mp18*sigA*, was used as a template for site-directed mutagenesis, as described above, with the mutagenic oligonucleotides listed in Table 3. The *sigA*-containing *Hin*dIII fragment prepared from replicative-form DNA of M13mp18*sigA* or its mutant derivatives was then inserted into the *Hin*dIII site of pAG58-*ble* (48), oriented to place the *sigA* coding sequence under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible P_{spac} promoter described by Yansura and Henner (45). To integrate the P_{spac}-*sigA*/*ble* cassettes into the *B. subtilis* chromosome in single copy, we constructed vectors (e.g., pTV21-SPIGMA [Fig. 1]) in which the expression cassettes were flanked by DNA sequences homologous to Tn*917*. This was accomplished by ligating gel-purified *Nco*I-*Pst*I fragments from pTV21Δ2 containing the two arms of Tn*917* to the 6.4-kb *Pst*I-*Nco*I fragment from pSPIGMA containing

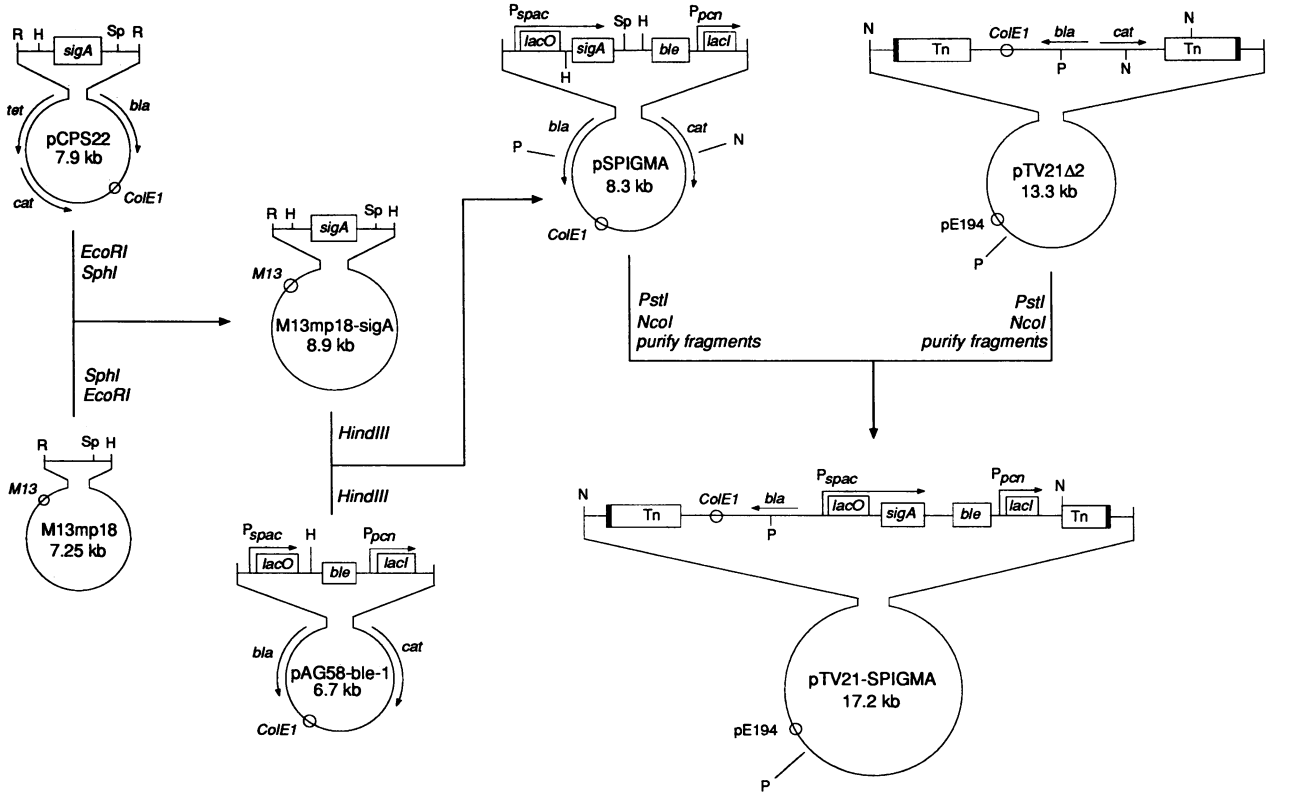

FIG. 1. Construction of a P_{spac}-*sigA* expression cassette flanked by Tn*917* arms. Plasmid pCPS22 (30) contains a cloned copy of the *B. subtilis rpoD* operon and is similar to pSCP2 (31), except that the *Eco*RI fragment containing *rpoD* (*sigA*) is in the reverse orientation. To construct M13mp18*sigA*, a 1.7-kb *Eco*RI-*Sph*I fragment including the promoterless *sigA* coding sequence was inserted into the *Eco*RI-*Sph*I backbone of M13mp18 (44). To place *sigA* under P_{spac} control, a 1.7-kb *Hin*dIII fragment from M13mp18*sigA* was inserted into the *Hin*dIII site of pAG58-*ble*-1 (48), creating pSPIGMA. To obtain pTV21-SPIGMA, the *Pst*I-*Nco*I fragment from pSPIGMA containing P_{spac}-*sigA* was combined for ligation with the two *Pst*I-*Nco*I fragments from pTV21Δ2 (47) that contain, respectively, the two arms of Tn*917*. This ligation mixture was then used to transform a *B. subtilis* strain already containing pTV21Δ2 to Ble^r. Abbreviations: R, *Eco*RI; H, *Hin*dIII; Sp, *Sph*I; P, *Pst*I; N, *Nco*I; *tet*, tetracycline resistance determinant from pBR325 (4); *bla*, β-lactamase gene from pBR325 (in the case of pCPS22) or pBR322 (5); *cat*, chloramphenicol resistance determinant from pBR325 (in the case of pCPS22) or from pC194; *ble*, bleomycin/phleomycin resistance determinant from pUB110 (36); Tn (pTV21Δ2 and derivatives), the arms of Tn*917*; ∅ ColE1, ∅ M13, ∅ pE194, replication origins derived from these plasmids or phages. Elements making up the SPAC expression cassette of Yansura and Henner (45): *lacI*, the *E. coli lac* repressor gene; *lacO*, the *E. coli lac* operator; P_{pcn}, a constitutive promoter driving expression of *lacI*; P_{spac}, a promoter derived from phage SPO1 which is IPTG inducible because of the close proximity of *lacO*.

the P_{spac}-*sigA*/*ble* cassette (Fig. 1). Such ligation mixtures were then used to transform PY231, a *B. subtilis* strain already containing pTV21Δ2, to Pm^r. The desired constructions thus arose as a product of recombination between ligated DNA and the resident plasmid. Vectors such as pTV21-SPIGMA were then used to transform KS160, which contains a phenotypically silent chromosomal insertion of Tn*917*, with a selection for Pm^r. Transformants arising from the kind of marker replacement event illustrated in Fig. 2 were identified by screening for Em^s.

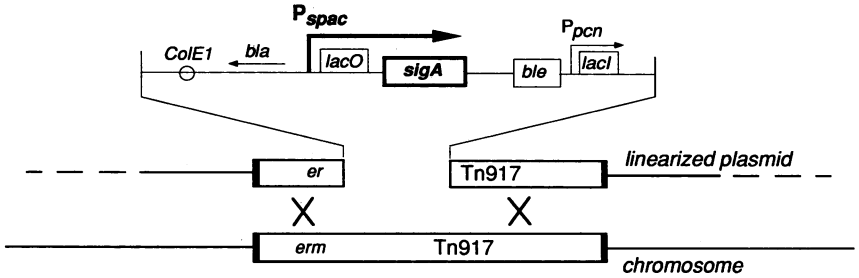

FIG. 2. Recombinational integration of the P_{spac}-*sigA* expression cassette into the chromosome. Illustrated schematically is the way in which Tn*917* homology can mediate recombinational integration of plasmids with the structure of pTV21-SPIGMA into a chromosomal copy of Tn*917*. Features of the structures shown are identified in the legend to Fig. 1. Plasmid linearization occurs in the natural course of DNA uptake by competent cells.

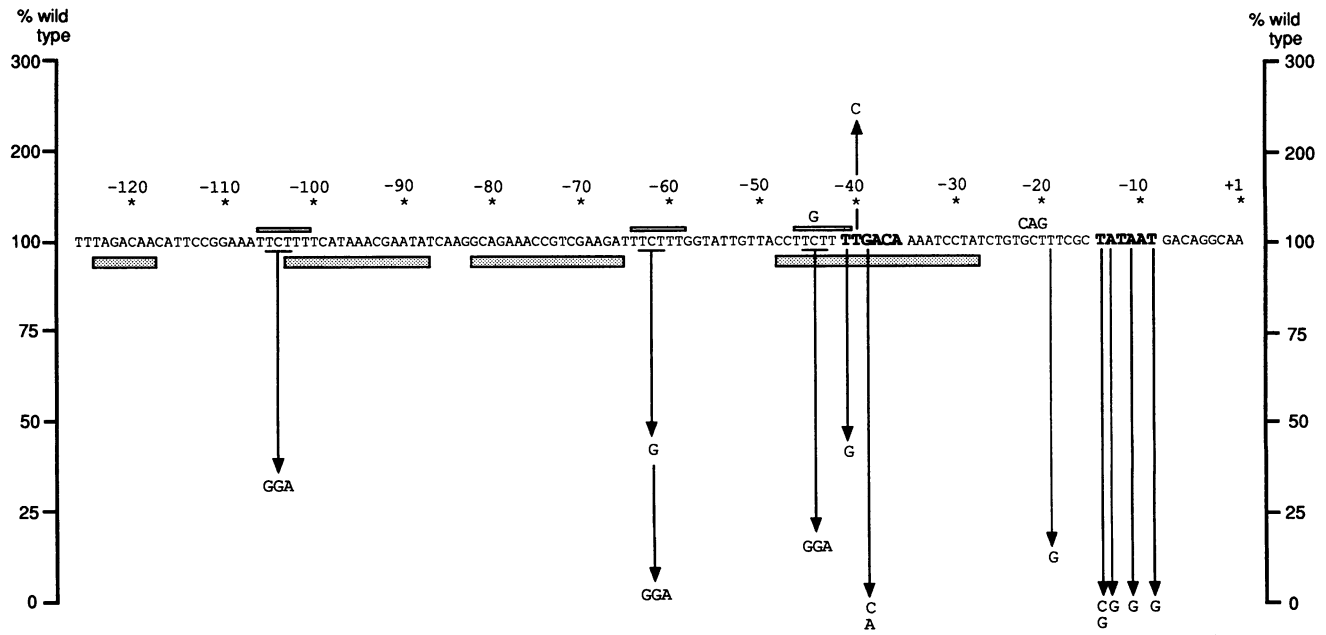

FIG. 3. Effects of site-directed mutations on transcriptional activity from the *spoIIE* promoter. The nucleotide sequence of the nontranscribed strand of the *spoIIE* regulatory region is shown to −126. The apparent start point of transcription as determined by primer extension is indicated as +1. Single or triple (underlined) base pair substitutions are indicated. β-Galactosidase levels in strains containing transcriptional *lacZ* fusion to the *spoIIE* promoter or its mutant derivatives were monitored at 30-min intervals during growth and sporulation in DSM. The arrows indicate the activity of each mutant promoter relative to that of the wild type. The values reflect the levels of β-galactosidase activity 4 h after the end of exponential growth and were derived from the average of at least two experiments, except for the −22 G-to-C change, which represents a single data set. Mutations which increase activity slightly over that of the wild type are shown just above the wild-type sequence. In boldface type are sequences which match the consensus for σ^A-associated RNA polymerase. Open overbars indicate sequences related to the motif TTCTTT. Stippled bars below the sequence indicate regions on the transcribed strand that were protected from DNase I digestion in the presence of Spo0A.

Preparation of RNA. Strains were grown at 37°C under moderate aeration in DSM (46) containing 5 μg of chloramphenicol per ml, 1 μg of erythromycin per ml, 25 μg of lincomycin per ml, and 0.5 μg of phleomycin per ml. At the end of exponential growth, the culture was divided into three equal samples. One sample was harvested immediately by centrifugation at 6,000 × *g* for 5 min at 4°C. This was designated the T_0 sample. The other two samples were incubated for an additional 3 h before harvesting, one in the presence of 1 mM IPTG and the other without IPTG. RNA was extracted from harvested cells by the guanidine isothiocyanate procedure described by Kenney et al. (16).

Primer extension analysis. Primer extension reactions were carried out as described by Moran (25), with 50-μg samples of template RNA, avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc.), and the oligonucleotide primers 5′-GTCCCGCCATTGGCCCG-3′ (complementary to *spoIIE* RNA and predicted to generate a 55-nucleotide extension product) and 5′-GGATGTGCTGCAAGGCG-3′ (complementary to *spoIIE-lacZ* RNA, annealing within the *lacZ* coding sequence, and predicted to generate a 195-nucleotide extension product when the template transcript initiates from the correct start site).

DNase I protection experiments. Uniquely end-labeled DNA fragments containing the *spoIIE* promoter were obtained as follows. Appropriate plasmid DNA samples were digested with *Eco*RI or *Hin*dIII to generate the ends to be labeled. Labeling was accomplished by filling in recessed 3′ ends with the Klenow fragment of DNA polymerase in the presence of [α-^{32}P]dATP (*Eco*RI) or both [α-^{32}P]dATP and [α-^{32}P]dGTP (*Hin*dIII) (>3,000 mCi/mmol; Amersham Co.). After digestion with the other enzyme (*Hin*dIII or *Eco*RI), the fragments were fractionated on 5% polyacrylamide gels. The fragments were eluted with 0.5 M ammonium acetate–1.0 mM EDTA at 37°C overnight and concentrated by ethanol precipitation. Spo0A protein was incubated with DNA as described by Satola et al. (35) with the Spo0A protein preparation described in that reference. Samples were subjected to DNase I digestion as described by Craig and Nash (7), with 5 μl of a 6.7-U/ml dilution of pancreatic DNase I (Worthington), for 30 s on fragment samples without protein and for 2 min on fragment samples to which Spo0A protein had been added.

RESULTS

Effects of mutations in the *spoIIE* regulatory region. The results of previous deletion analysis indicated that a segment of DNA extending at least −112 bp upstream of the transcription start site was required for full expression and regulation of the *spoIIE* promoter (11). To determine which sequences within this segment were important, we introduced site-directed mutations and tested them for possible effects on *spoIIE* transcription (Fig. 3) by constructing strains containing an SPβ phage-borne transcriptional *lacZ* fusion to the *spoIIE* promoter or its mutant derivatives and monitoring β-galactosidase activity during growth and sporulation in DSM.

To evaluate the possible significance of sequences which resemble σ^A recognition hexamers, we introduced mutations

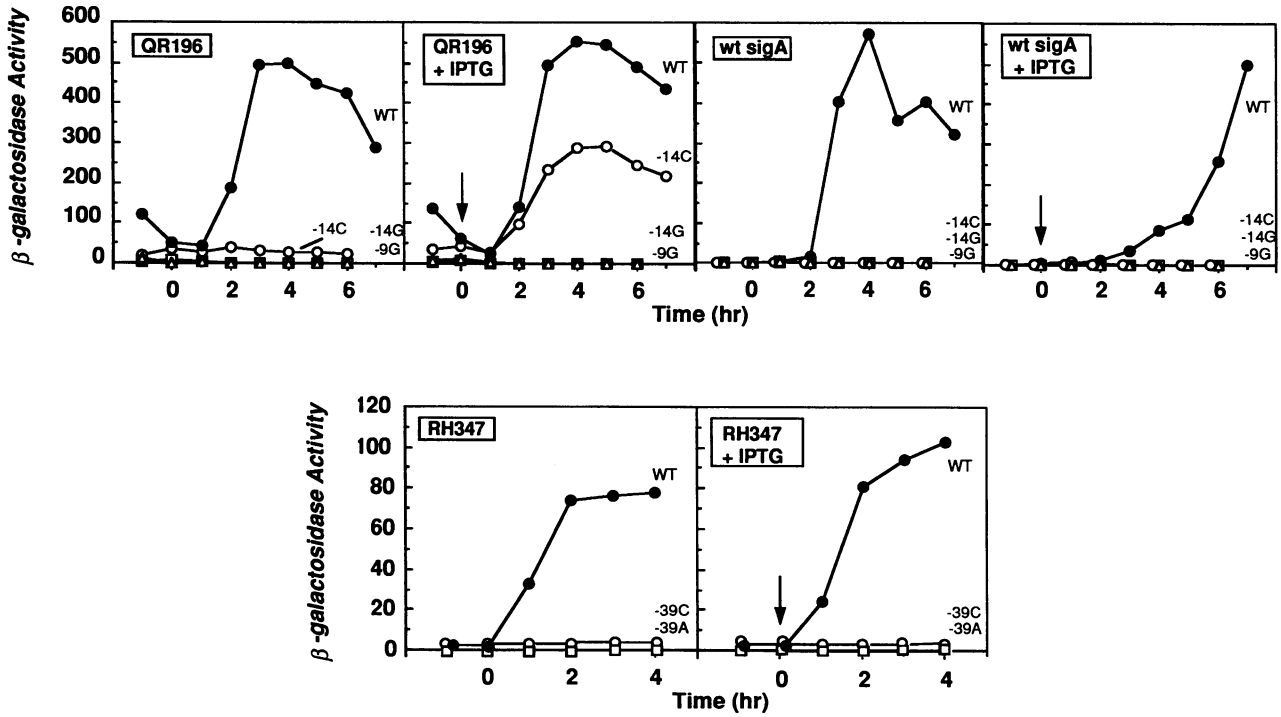

FIG. 4. Effects on mutant *spoIIE* promoters of IPTG-induced expression of mutant *sigA* genes. Top four panels: ●, wild-type *spoIIE-lacZ* fusion; ○, −14C; □, −14G; △, −9G; assays were carried out and activity was calculated by the method of Youngman (46). Bottom two panels: ●, wild-type *spoIIE* promoter; ○, −39A; □, −39C; assays were carried out and activity was calculated by the method of Miller (24). For all experiments, the version of *sigA* provided under P_{spac} control is indicated in the boxed inset; +IPTG, the addition of IPTG to a final concentration of 1.0 mM at the time indicated by the arrow.

that changed these sequences away from the σ^A consensus. Five such mutations introduced into the −10 element all reduced transcriptional activity to less than 5% of the wild-type activity (Fig. 3), consistent with the possibility that these elements act as polymerase recognition sequences. Three mutations (G to C at −39, G to A at −39, and T to G at −41) introduced into the −35-like element had a similar effect (Fig. 3). Of particular interest were the mutations at position −39, which abolished all detectable transcription activity (Fig. 4). However, a fourth mutation in the −35-like element (T to C at −40) actually increased promoter activity, although it also represents a change away from the consensus for an $E\sigma^A$ recognition sequence. The same kind of anomaly had previously been observed with a mutation at the analogous position in the *spoIIG* promoter (13).

The occurrence of a TTCTTT motif at three locations in the *spoIIE* regulatory region had also been noted previously (11). To evaluate the possible regulatory significance of elements containing this motif, we disrupted them by single-base substitutions or by blocks of three-base changes, introduced by site-directed mutagenesis. Most of these mutations reduced promoter activity to some extent. The most extreme effect was observed with a 3-bp substitution at −63 to −61, which reduced transcriptional activity to less than 1% of the wild-type activity. A single-base change in this element (C to G at −63) reduced activity to 55% of wild-type activity, whereas the analogous mutation at −43 had no effect.

Four mutations were introduced into the spacer region between the −10 and −35-like elements, altering sequences which are found in both the *spoIIE* and *spoIIG* promoters. Although mutation of T to G at −19 reduced promoter activity, three other mutations (T to G at −20, C to A at −21, and G to C at −22) increased promoter activity slightly above the wild-type activity. The significance of these results is not yet understood, but mutations at the analogous positions in the *spoIIG* promoter have similar effects (35).

Evidence that the *spoIIE* promoter is recognized by $E\sigma^A$. To determine whether $E\sigma^A$ recognizes the *spoIIE* promoter in vivo, site-directed mutations were introduced into *sigA* (*rpoD*) and evaluated for their ability to suppress the negative effects of mutations in the −10 or −35-like region of the *spoIIE* promoter. The design of this experiment was guided by the results of studies of allele-specific suppression in *E. coli* (9, 37, 42) and based on the observation that the amino acid sequences of *E. coli* σ^{70} and *B. subtilis* σ^A believed to make direct contact with the −10 and −35 regions of promoter sequences are virtually identical (15, 31). In addition, because *sigA* is an essential gene and because we suspected that change-of-specificity *sigA* alleles might be dominant-lethal mutations, *sigA* partial diploids were constructed for these experiments in which expression of the mutant forms of *sigA* was placed under control of the IPTG-inducible P_{spac} promoter. P_{spac}-driven mutant alleles of *sigA* were integrated into the *B. subtilis* chromosome in single copy by constructing a plasmid in which Tn*917* arms flanked the P_{spac}-*sigA* cassette (Fig. 1). This made it possible to integrate P_{spac}-*sigA* cassettes by homologous recombination at the site of existing Tn*917* chromosomal insertions (Fig. 2).

In agreement with results obtained previously with the *spoIIG* promoter (16), a mutation which produced a glutamine-to-arginine substitution at amino acid 196 (QR196) in *sigA* was able to compensate for a TATAAT-to-CATAAT mutation in the −10 hexamer of the *spoIIE* promoter (Fig.

4). Compensation was IPTG dependent, suggesting that it was mediated directly by the mutant SigA protein. The result was allele specific with respect to the mutant promoter, since two other −10 hexamer substitutions, including a different substitution at the same position, were not compensated (Fig. 4). The effect was also allele specific with respect to *sigA*, since neither of two other mutant alleles of *sigA*, nor a wild-type copy of *sigA* under P_{spac} control, was able to suppress any of the promoter mutations tested (Fig. 4) (data not shown). This suggests that the *spoIIE* promoter, like the *spoIIG* promoter, is utilized by $E\sigma^A$ and that σ^A protein interacts directly with the −10 regions of these promoters.

If the QR196 *sigA* mutation causes a true change-of-specificity alteration in the SigA protein, transcription from the mutant promoter containing the CATAAT substitution should initiate at the same start site as the normal *spoIIE* transcript. To test this, we determined transcription start sites by primer extension, using two primers (Fig. 5). One primer annealed within the *lacZ* coding sequence and was thus specific for transcripts initiating from the phage-borne *spoIIE* promoter driving expression of the *lacZ* gene. With this primer, the results confirmed that in a strain containing the QR196 *sigA* allele, IPTG induced a transcript from the promoter containing the −14 T-to-C mutation that initiated at the same nucleotide (Fig. 5, lanes b and c) as the transcript generated by wild-type $E\sigma^A$ from the wild-type promoter (lanes e and f). With a primer that detected transcripts initiating from the normal chromosomal *spoIIE* promoter as well as the phage-borne *spoIIE* promoter, IPTG induced no new transcript (lanes h, i, k, and l). In addition to confirming that IPTG-induced transcription initiated from the correct start site, these results explained another observation. A significant level of vegetative expression of *lacZ* was observed in some strains that contained mutant *sigA* alleles (Fig. 4). Lane g of Fig. 5 indicates that this is adventitious transcription initiating from a location upstream of the normal transcription start site.

To investigate whether σ^A also contacts the "−35 region" of the *spoIIE* promoter, an allele of *sigA* containing an arginine-to-histidine change at amino acid 347 (RH347) was tested for its ability to suppress a change from TTGACA to either TTAACA or TTCACA in the −35-like hexamer. In two other promoters known to be transcribed by $E\sigma^A$ in *B. subtilis*, both of these −35 substitutions were previously shown to be suppressed by the RH347 form of SigA (15). In the *spoIIE* promoter, however, no suppression was seen (Fig. 4). A similar result was obtained for the *spoIIG* promoter in earlier work (16). This suggests, at least, that $E\sigma^A$ utilizes the −35-like hexamer in the *spoIIE* and *spoIIG* promoters in a way that differs from its mode of interaction with conventional vegetative promoters; it is consistent with the interpretation that the −35-like hexamer of the *spoIIE* and *spoIIG* promoters is not utilized at all in any direct way by $E\sigma^A$.

DNase I protection of the *spoIIE* promoter by Spo0A protein. Spo0A is a regulatory protein believed to control the initiation of sporulation by activating or repressing specific promoters (35, 41). Evidence was recently reported that Spo0A may stimulate transcription from the *spoIIG* promoter by binding to sites conforming to the consensus sequence 5′-TG(A/T)CGAA-3′ (35). Spo0A also acts as a negative regulator at some promoters by binding to a similar, if not identical, site (41). Inspection of the *spoIIE* promoter region revealed four sites resembling this proposed Spo0A-binding sequence. Interestingly, one of these sites overlaps the −35-like sequence and actually provided a potential

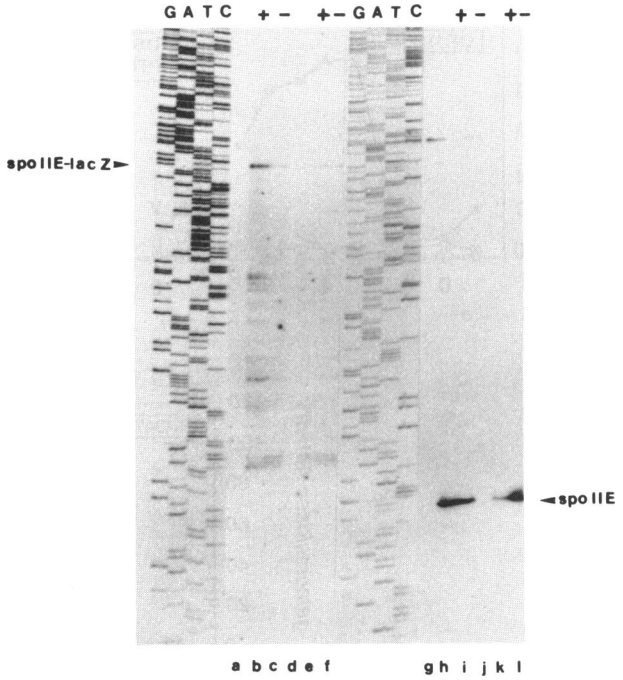

FIG. 5. Primer extension analysis of *spoIIE-lacZ* transcripts. RNA was isolated from two strains that contain the QR196 allele of *sigA* under the control of P_{spac}. KY84 (lanes d to f and j to l) contains a phage-borne wild-type *spoIIE* transcriptional *lacZ* fusion, and KY83 (lanes a to c and g to i) contains a phage-borne transcriptional *lacZ* fusion to the −14C-*spoIIE* mutant promoter. Strains were grown in DSM, and cells were harvested for RNA extraction at T_0 (lanes a, d, g, and j) or T_3 in the presence (+) (lanes b, e, h, and k) or absence (−) (lanes c, f, i, and l) of IPTG, as described in Materials and Methods. A radiolabeled primer complementary to the *lacZ* coding region (indicated as *spoIIE-lacZ*) (lanes a to f) or *spoIIE* (indicated as *spoIIE*) (lanes g to l) was annealed to the RNA and extended by using avian myeloblastosis virus reverse transcriptase. For each primer, an arrowhead indicates the expected size for a primer extension product initiating from the previously determined start point of transcription. The same oligonucleotides were used to prime dideoxy sequencing reactions from a single-stranded DNA template, M13mp18-9 (Table 2), which contains the promoter-proximal portion of a *spoIIE-lacZ* fusion (left), or M13mp18-8 (Table 2), which contains the *spoIIE* regulatory region (middle). The letters above the lanes indicate the dideoxynucleotide used to terminate each reaction. This figure is a composite of two autoradiographic exposures of the same gel.

explanation for the anomalous transcription enhancement phenotype exhibited by the T-to-C promoter mutation at −40; although this change is away from the consensus for σ^A-like −35 sequences, it is toward the proposed consensus for Spo0A-binding sites (Fig. 6). This prompted us to determine whether Spo0A could bind to the *spoIIE* regulatory region in vitro and to examine the effects of −35 hexamer mutations on binding. When end-labeled DNA corresponding to the transcribed strand of the *spoIIE* regulatory region was used, addition of increasing amounts of a partially purified protein preparation containing Spo0A protected four sites against DNase I digestion (Fig. 7); each protected region contained a sequence identified by inspection of the promoter region as a potential Spo0A-binding site (see Fig. 3). As indicated in Fig. 7, we have designated these protected regions sites A through D; site A overlaps the −35-like sequence.

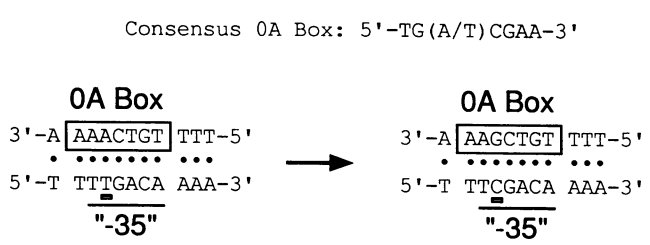

FIG. 6. Consequences of a T-to-C mutation at position −40. At the left is the sequence of a portion of the wild-type *spoIIE* promoter, from −43 to −33, which includes the −35-like element; bases at −40 on the coding strand are underscored by an open rectangle. Seven bases on the template strand resembling the "0A box" proposed by Strauch et al. (41) are highlighted by an open box. As shown, the T-to-C substitution at −40 creates a perfect match to the 0A box consensus.

The effects of three promoter mutations on the pattern of Spo0A binding were also examined. These included two mutations in the *spoIIE* regulatory region that eliminated transcription and one mutation (−40 T to C) that increased promoter activity. When DNA containing a 3-bp substitution of TTCTTT-to-TGGATT positioned at −63 to −61 in the *spoIIE* regulatory region was used, the pattern of DNase I

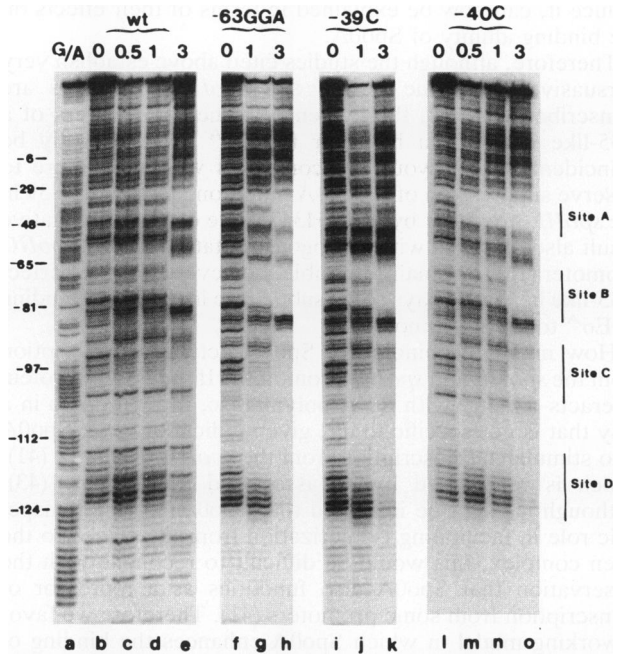

FIG. 7. DNase I protection of the *spoIIE* regulatory region by Spo0A. A 274-bp *Eco*RI-*Hin*dIII DNA fragment containing the *spoIIE* regulatory region was 3′ end labeled at the *Eco*RI site (template strand). This DNA was digested with DNase I after incubation in the presence or absence of partially purified Spo0A as described in Materials and Methods. The amount of total protein in each reaction (in micrograms) is indicated above each lane. The products of chemical sequencing reactions for A and G (lane a) were used as molecular size markers. The positions of cleavage products relative to the start point of transcription are indicated at the left. Lanes: b to e, wild-type DNA; f to h, DNA containing the 3-bp substitution TCT to GGA at −61 to −63; i to k, DNA containing the −39 G-to-C mutation; lanes l to o, DNA containing the −40 T-to-C mutation. The vertical bars indicate the regions of protection (sites A through D).

protection was not significantly different from that observed with wild-type DNA, except perhaps to enhance protection somewhat at all four binding sites (Fig. 7, lanes f to h). This mutation had greatly reduced transcription from the *spoIIE* promoter in *lacZ* fusion experiments but was not within one of the protected sites (Fig. 3). In contrast, the pattern of DNase I protection was significantly altered when DNA containing a −39 G-to-C mutation was used (lanes i to k). This mutation reduced Spo0A binding at site A, whereas sites B through D were protected to an extent indistinguishable from that of the wild type. When using DNA containing a −40 T-to-C mutation, a mutation in site A which increased transcription from the mutant promoter in vivo, site A was preferentially protected at a lower concentration of protein. Thus the binding pattern in vitro for two point mutations in site A correlates with their opposite effects on promoter activity in vivo. We take this as evidence that Spo0A rather than $E\sigma^A$ interacts with the −35-like sequence and that the binding of Spo0A somehow enables $E\sigma^A$ to utilize this promoter during sporulation.

The pattern of genetic dependencies supports a direct interaction of Spo0A with *spoIIE*. Mutations in *spo0A* were shown to block expression of *spoIIE* in previous work (11). However, Spo0A affects many genes indirectly by negatively regulating expression of the *abrB* gene, a repressor of some stationary-phase genes (28, 50). Therefore, mutations in *spo0A* block expression of such *abrB*-repressed genes, but this block is relieved in an *abrB spo0A* double mutant. To address the possibility that Spo0A acts indirectly on the *spoIIE* promoter (i.e., through AbrB), we examined *spoIIE* expression in an *abrB spo0A* double mutant. The results (Fig. 8A) show that introduction of an *abrB* mutation fails to enhance *spoIIE* expression in a *spo0A* mutant. Similar results have been reported for *spoIIG* (14) and *spoIIA* (43). Two other early-blocking mutations whose effects on *spoIIE* were not tested in previous work include *kinA* (*spoIIJ*) and *spoIIF*. Although the function of *spoIIF* in sporulation is not yet understood, *kinA* is thought to be part of a multicomponent phosphorelay system which activates Spo0A during sporulation (1, 6). As shown by the results in Fig. 8, mutations in both of these loci significantly reduce *spoIIE* expression. Thus all available genetic evidence is consistent with a direct interaction of Spo0A with the *spoIIE* promoter.

Temporal expression patterns of *spoIIE*, *spoIIG*, and *spoIIA*. Recent work has implicated Spo0A as a transcriptional activator of three temporally regulated stage II operons, *spoIIG* (35), *spoIIA* (6), and *spoIIE* (this report). Although the temporal expression patterns of all three operons were examined in previous work (11, 14, 29), strain differences and variations in assay protocols make it difficult to evaluate apparent differences in times of induction. To facilitate a direct comparison, transcriptional *lacZ* fusions to *spoIIA*, *spoIIE*, and *spoIIG* were transferred to isogenic strains and assayed simultaneously. When the results were plotted as a percentage of maximum expression for each fusion, time courses of induction for the three fusions were found to be indistinguishable (Fig. 9). These results are in agreement with results previously reported by Stragier et al. (39) for *spoIIA* and *spoIIG*.

DISCUSSION

The hypothesis that $E\sigma^A$ directs the transcription of the *spoIIE* and *spoIIG* operons originated with the discovery of sequences immediately upstream from their transcription start sites that closely resemble recognition hexamers lo-

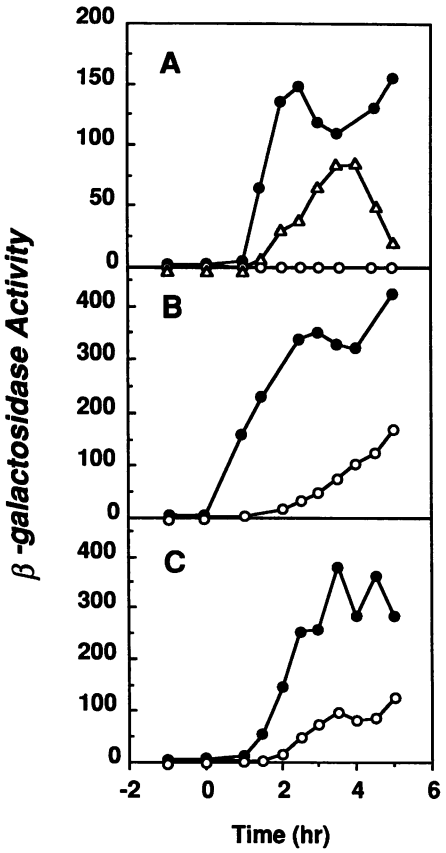

FIG. 8. Effects of sporulation-related mutations on the activity of a *spoIIE-lacZ* transcriptional fusion. Wild-type and mutant strains were cultured in DSM and sampled for β-galactosidase activity during growth and sporulation as described in Materials and Methods. Time zero indicates the end of exponential growth. (A) Symbols: ●, wild type (PY309); ○, *spo0A abrB* double mutant (KY269); △, *abrB* mutant (KY265). A strain isogenic to KY269 but containing only a *spo0A* mutation (KY261) was assayed simultaneously and found to produce only background activity similar to that of KY269 (data not shown). (B) Symbols: ●, wild type (PY309); ○, *kinA* (*spoIIJ*) mutant (KY277). (C) Symbols: ●, wild type (KY281); ○, *spoIIF* mutant (KY282). Activity was determined by the method of Youngman (46).

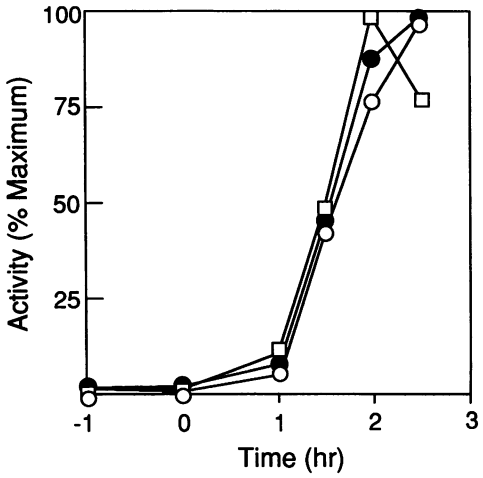

FIG. 9. Temporal expression patterns of *spoIIE-lac*, *spoIIG-lac*, and *spoIIA-lacZ* fusions in isogenic strains. Symbols: ●, *spoIIE-lacZ* (KY21); ○, *spoIIG-lacZ* (KY255); □, *spoIIA-lacZ* (KY20). Activity was determined by the method of Youngman (46).

cated in the −10 and −35 regions of promoters utilized by *E. coli* $E\sigma^{70}$ and *B. subtilis* $E\sigma^{A}$ (11, 14). This was especially striking for *spoIIE*, which contained sequences showing a perfect match to both the −10 and −35 consensus sequences. The results of in vitro transcription studies (13), site-directed mutagenesis (35; this work) and allele-specific suppression analysis (16; this work) strongly supported the conclusion that both the *spoIIE* and *spoIIG* promoters were utilized by $E\sigma^{A}$. Nevertheless, the inappropriate spacing between the −35-like and −10-like sequences in these promoters was difficult to accommodate in a straightforward model; a spacing of 21 or 22 bp placed the −35 hexamer on the wrong side of the helix relative to the −10 hexamer. This seemed to suggest the involvement of an additional regulatory factor that might distort either the σ^{A} protein itself or the local structure of the DNA helix in the promoter region to compensate. Evidence presented here and in a separate report (35) that the −35-like sequences within the *spoIIE* and *spoIIG* promoters are contained within a biologically active binding site for the Spo0A protein resolves this paradox. The effects of all mutations within this −35-like sequence, including mutations that enhance expression as well as those that reduce it, can now be explained in terms of their effects on the binding affinity of Spo0A.

Therefore, although the studies cited above establish very persuasively that the *spoIIE* and *spoIIG* promoters are transcribed by $E\sigma^{A}$, the presence in these promoters of a −35-like recognition hexamer for $E\sigma^{A}$ could actually be coincidental. This would be consistent with our failure to observe suppression of a G-to-A mutation at position −39 in the *spoIIE* promoter by the RH347 allele of *sigA*, a negative result also obtained with analogous mutations in the *spoIIG* promoter (16). It remains possible, however, that the perfect −35-like hexamer plays some subtle role in the initial binding of $E\sigma^{A}$ to these promoters.

How might the binding of Spo0A activate transcription from the *spoIIE* and *spoIIG* promoters? If the Spo0A protein interacts directly with RNA polymerase, it must do so in a way that is not specific to σ^{A}, given indications that Spo0A also stimulates transcription from the *spoIIA* promoter (41), which is recognized by σ^{H}-associated holoenzyme (43). Although it might be imagined that Spo0A plays a nonspecific role in facilitating isomerization from the closed to the open complex, this would be difficult to reconcile with the observation that Spo0A also functions as a repressor of transcription from some promoters (41). Therefore we favor a working model in which Spo0A enhances the binding of RNA polymerase, perhaps through a direct interaction with the polymerase core that takes place after Spo0A is bound to its own recognition site. It is also possible that Spo0A makes direct contact with a conserved domain of σ.

On the basis of available evidence regarding Spo0A specifically (6, 27), and considering known properties of better-characterized members of "two-component" systems of bacterial signal transduction proteins (38), it is reasonable to speculate that Spo0A requires phosphorylation in vivo to stimulate transcription from the *spoIIE* and *spoIIG* promoters. Whether phosphorylation affects the binding of Spo0A to its target sites in the *spoIIE* or *spoIIG* promoter is not known. Therefore some caution should be exercised in drawing strong conclusions from in vitro binding studies,

such as those reported here, which use Spo0A purified from a strain of *E. coli*. Although we have not assessed the phosphorylation state of Spo0A prepared in this way, it seems likely that very little of it would be in the phosphorylated form.

What role does Spo0A play in the temporal regulation of the *spoIIE* and *spoIIG* operons? Both operons are transcriptionally silent or expressed at very low levels during growth and become active about 60 to 90 min after T_0. Spo0A is believed to be activated by phosphorylation at around T_0 (6, 27). Therefore, transcriptional silence of the *spoIIE* and *spoIIG* operons prior to T_0 might simply reflect the absence of phospho-Spo0A. However, the kinetics of *spoIIE* and *spoIIG* activation following T_0 suggest that the appearance of phospho-Spo0A is probably not sufficient to enable transcription to occur. Phosphorylation of Spo0A is also believed to (indirectly) activate *spoVG* transcription (40, 49), which occurs about 1 h earlier than the activation of *spoIIE* and *spoIIG*. This implies either that the levels of phospho-Spo0A required for induction of *spoVG* are different from those required for induction of *spoIIE* and *spoIIG* or that an additional factor is involved in the activation of *spoIIE* and *spoIIG* transcription. That additional factor could be the Sin protein described by Gaur et al. (10), which appears to be a repressor of several stage II transcription units, including *spoIIA*, *spoIIE*, and *spoIIG* (21).

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We gratefully acknowledge advice and suggestions from R. Losick, who proposed the use of *sigA* mutants as a possible way to establish whether the σ^A protein is responsible for utilization of the *spoIIE* and *spoIIG* promoters in vivo. We also thank I. Smith for many helpful discussions.

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