JOURNAL OF BACTERIOLOGY, Apr. 1988, p. 1598–1609
0021-9193/88/041598-12$02.00/0

Vol. 170, No. 4

Characterization of the Promoter Region of the *Bacillus subtilis spoIIE* Operon

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Received 9 November 1987/Accepted 25 January 1988

Mutations that define the *spoIIE* locus of *Bacillus subtilis* block sporulation at an early stage and recently were shown to prevent the proteolytic processing of σ^E (σ^{29}) into its active form, an event that is believed to control critical changes in gene expression during the second hour of development. By taking advantage of two Tn*917*-mediated insertional mutations in *spoIIE*, we have cloned DNA spanning the locus. Gene disruption experiments with subcloned fragments transferred to integrational vectors revealed that the locus consisted of a single transcription unit about 2.5 kilobase pairs in size. Transcriptional *lacZ* fusions were used to show that expression of this transcription unit initiated at 1.5 h after the end of log-phase growth and depended upon the products of all *spo0* loci. Expression was directed by a single promoter whose position was determined by high-resolution S1 protection mapping. A deletion analysis of the promoter region was also carried out, with novel integrational vectors based on derivatives of coliphage M13. The results indicated that a region of DNA extending from 183 to 118 base pairs upstream from the start point of transcription was required for full activity of the *spoIIE* promoter. The presumptive RNA polymerase-binding region of the promoter exhibited striking similarity to the *spoIIG* promoter and featured perfect but unusually spaced −10 and −35 consensus sequences for σ^A (σ^{43})-associated RNA polymerase.

Endospore formation in *Bacillus subtilis* involves an elaborate program of morphological development and is accompanied by complex changes in gene expression (19). Mutations that prevent spore formation, *spo* mutations, define approximately 50 genetic loci and generally block morphological development at a particular stage (32). Many *spo* loci consist of polycistronic operons that include more than one gene whose product is essential for sporulation (20). Moreover, with a surprisingly high frequency, *spo* mutations have identified genes or operons whose products apparently play regulatory roles (20). This finding may reflect the possibility that only highly pleiotropic mutations arrest development at discrete stages, and these mutations may be predominantly those affecting regulatory genes.

The first obvious morphological manifestation of sporulation is the formation of an asymmetric cell septum that partitions the cell into two compartments of unequal size. This is referred to as stage II. In the course of normal development, this asymmetric septum migrates toward the pole of the cell associated with the smaller of the two cell compartments, ultimately engulfing the smaller compartment completely. Mutations that arrest development at stage II, *spoII* mutations, define at least nine loci. These include *spoIIA*, *spoIIB*, *spoIID*, *spoIIE*, *spoIIF*, and *spoIIG*, which were identified in earlier genetic studies (32), and *spoIIJ*, *spoIIL*, and *spoIIM*, which were identified recently as the result of Tn*917*-mediated insertional mutagenesis (37).

The genetic organization and transcriptional regulation of several *spoII* loci have been investigated in recent work. Consistent with the pattern noted above, the studied loci in all cases encode at least some gene products that probably perform regulatory functions. For example, the *spoIIG* locus consists of a polycistronic operon which includes at least two genes essential for sporulation (15). One of these genes, corresponding to the second open reading frame of the operon, is *sigE*, which encodes the 31-kilodalton precursor of σ^E (formerly σ^{29}) (17, 39, 40). The *spoIIG* operon becomes transcriptionally active at the onset of sporulation (15), and P^{31}, the primary gene product of *sigE*, can be detected immunologically within the first 30 min of sporulation (41). However, the proteolytic processing of P^{31} does not take place until the second hour of sporulation and depends upon the products of the *spoIIE* locus (41), as well as upon the gene product of the promoter-proximal open reading frame of *spoIIG* (14). The *spoIIA* locus also consists of a polycistronic operon (9, 33). This operon becomes transcriptionally active at 60 to 90 min after the onset of sporulation and, like *spoIIG*, also includes at least two genes essential for sporulation, *spoIIAA* and *spoIIAC* (8, 31). The gene product of *spoIIAC* exhibits strong homology to the characterized σ factors of *B. subtilis* and *Escherichia coli* and is presumed either to function as a σ factor itself or to play a regulatory role that requires binding to the RNA polymerase core in the way σ factors do (7). Phenotypes of mutations in *spoIIAA* suggest that the gene product of *spoIIAA* may act in conjunction with the product of *spoIIAC* (P. J. Piggot, personal communication). As with *spoIIG*, expression of the *spoIIA* operon is unaffected by mutations in any *spoII* locus (8, 31). The *spoIID* locus is associated with a monocistronic transcription unit that becomes active at about 2 h after the end of log-phase growth (T_2) (4, 18, 34). Expression of *spoIID* depends upon the products of *spoIIA*, *spoIIE*, and *spoIIG* (4, 34). It has been inferred from the DNA sequence of the *spoIID* locus that the gene product of *spoIID* is probably a DNA-binding protein that may perform a regulatory function (12).

In the present work, we have investigated the transcriptional organization and regulation of the *spoIIE* locus. We found that this locus consisted of a single transcription unit of at least 2.5 kilobase pairs (kb) in size. It had a single promoter which depended upon a DNA interval extending more than 118 base pairs (bp) upstream from the start point of transcription for full activity. This promoter became active at $T_{1.5}$ and was independent of the products of all

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TABLE 1. *B. subtilis* strains used

Strain	Genotype or relevant characteristics	Source or reference
BD170	*trpC2 thrA5*	D. Dubnau
JH646	*trpC2 pheA1 spo0A12*	J. A. Hoch
JH647	*trpC2 pheA1 spo0E11*	J. A. Hoch
JH648	*trpC2 pheA1 spo0B136*	J. A. Hoch
JH649	*trpC2 pheA1 spo0F221*	J. A. Hoch
JH695	*trpC2 pheA1 spo0C9V*	J. A. Hoch
PY367	*trpC2 spo0K141*	P. Zuber
IS223	*trpC2 spo0HΔHind*	I. Smith
IS33	*trpC2 rpoB2 spoIID66*	P. J. Piggot
SL401	*trpC2 spoIIAC1*	P. J. Piggot
SL493	*trpC2 metC3 rpoB2 spo0J93*	P. J. Piggot
SL617	*leu-8 tal-1 spoIIG55*	P. J. Piggot
SL731	*trpC2 spoIIB131*	P. J. Piggot
SA127	*pheA1 spoIIE64*	M. Young
PY180	*spoIIE*::Tn*917*ΩHU7	37
KS181	*spoIIE*::Tn*917*ΩHU181	37
ZB307	Lysogen SBβ*c2del2*::Tn*917*::pSK10Δ6	49
PY415	*spoIIE*::Tn*917*ΩHU181::*lac55*	This work[a]
PY436	*spoIIE*::Tn*917*ΩHU181::*lac55*::*erm32*	This work
GV1	Chr::pZΔ326-GV39 *trpC2 thrA5*	This work
GV2	Chr::M13mp19*cat*-1 *trpC2 thrA5*	This work
GV3	Chr::pZΔ326-GV39 *trpC2 spo0J93*	This work
GV4	Chr::pZΔ326-GV39 *trpC2 spoIIAC1*	This work
GV11	Chr::pZΔ326-GV39 *trpC2 spoIIG55*	This work
GV13	Chr::pZΔ326-GV39 *trpC2 spoIIB131*	This work
GV14	Chr::pZΔ326-GV39 *trpC2 spo0K141*	This work
GV15	Chr::pZΔ326-GV39 *trpC2 spo0C9V*	This work
GV16	Chr::pZΔ326-GV39 *trpC2 spo0F221*	This work
GV17	Chr::pZΔ326-GV39 *trpC2 spoIID66*	This work
GV18	Chr::pZΔ326-GV39 *trpC2 spo0E11*	This work
GV19	Chr::pZΔ326-GV39 *trpC2 spo0A12*	This work
GV22	Chr::pZΔ326-GV39 *trpC2 spo0B136*	This work
GV23	Chr::pZΔ326-GV39 *trpC2 spo0HΔHind*	This work
GV25	*spoIIE*::Tn*917*ΩHU181::*lac55*::*erm32*::M13mp19*cat*-19	This work
GV26	*spoIIE*::Tn*917*ΩHU181::*lac55*::*erm32*::M13mp19*cat*-4	This work
GV27	*spoIIE*::Tn*917*ΩHU181::*lac55*::*erm32*::M13mp19*cat*-29	This work
GV29	*spoIIE*::Tn*917*ΩHU181::*lac55*::*erm32*::M13mp19*cat*-17	This work
GV50	*spoIIE*::Tn*917*ΩHU181::*lac55*::*erm32*::M13mp19*cat*-32	This work
GV56	Lysogen SPβ*c2del2*::Tn*917*pSK10Δ6::pZΔ326-GV43	This work
GV57	Lysogen SPβ*c2del2*::Tn*917*pSK10Δ6::pZΔ326-GV49	This work
GV58	Lysogen SPβ*c2del2*::Tn*917*pSK10Δ6::pZΔ326-GV39	This work

[a] When source is this work, construction of strains is described in Table 2.

known *spoII* loci. The presumptive RNA polymerase-binding region of the *spoIIE* promoter displayed considerable similarity to the corresponding region of the *spoIIG* promoter. We consider the possible significance of unusually spaced consensus sequences in both promoters for σ^A (σ^{43})-associated RNA polymerase.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains used in this work are listed in Table 1. Strain constructions are summarized in Table 2. Plasmids and bacteriophages used in this work are listed in Table 3, together with descriptions of their sources or derivations.

Culture media and genetic techniques. LB medium, prepared as described previously (45), was used for routine culture of both *B. subtilis* and *E. coli* bacteria. TSS medium, prepared as described previously (45), was used as the synthetic minimal medium for *B. subtilis*, and M9 medium, prepared as described by Miller (26), was used for *E. coli*. DSM medium, prepared as described previously (45), was used as the sporulation medium for *B. subtilis*. Transformations of *B. subtilis* strains were carried out as described by Anagnostopoulos and Spizizen (1). Transformations or transfections of *E. coli* strains were carried out as described by Mandel and Higa (21). Selections for antibiotic resistances were carried out as described previously (45). Techniques for propagating M13 constructions as phage in *E. coli* and for isolating virion or replicative-form I (RFI) DNAs were as described by Messing (25). Techniques for the use of *B. subtilis* phage SPβ were as described by Rosenthal et al. (36).

In vitro manipulation of DNA. Chromosomal DNA was isolated from *B. subtilis* strains by a modification of the method of Marmur (22). Lysozyme-treated bacteria were lysed by the addition of Sarkosyl (CIBA-GEIGY Corp.) to a final concentration of 1% and extracted with an equal volume of phenol-chloroform. DNA was spooled from the aqueous phase, washed with 70% ethanol, and rinsed with ethyl ether. Preparative isolation of plasmid DNA from both *E. coli* and *B. subtilis* was carried out by the alkaline lysis procedure of Birnboim and Doly (3). Restriction enzymes, DNA ligase, alkaline phosphatase, S1 nuclease, polynucleotide kinase, and the Klenow fragment of DNA polymerase were purchased from Bethesda Research Laboratories, Inc., and used as instructed by the supplier. DNA-sequencing reactions were carried out either by the method of Maxam

TABLE 2. Construction of strains

Strain	Construction
PY415	Transformation of KS181 to Cm^r with pTV55 DNA that had been linearized by digestion with *PstI* (see Materials and Methods)
PY436	Transformation of PY415 to MLS^r with pTV32 DNA that had been linearized by digestion with *PstI* (see Materials and Methods)
GV1	Transformation of BD170 to Cm^r with pZΔ326-GV39
GV2	Transformation of BD170 to Cm^r with M13mp19*cat*-1 RFI DNA
GV3	A Spo^- transformant resulting from the transformation of GV1 to Thr^+ with a saturating concentration of chromosomal DNA prepared from SL493
GV4	As GV3, but with chromosomal DNA prepared from SL401
GV11	As GV3, but with chromosomal DNA prepared from SL617
GV13	As GV3, but with chromosomal DNA prepared from SL731
GV14	As GV3, but with chromosomal DNA prepared from PY367
GV15	As GV3, but with chromosomal DNA prepared from JH695
GV16	As GV3, but with chromosomal DNA prepared from JH649
GV17	As GV3, but with chromosomal DNA prepared from IS33
GV18	As GV3, but with chromosomal DNA prepared from JH647
GV19	As GV3, but with chromosomal DNA prepared from JH646
GV22	As GV3, but with chromosomal DNA prepared from JH648
GV23	As GV3, but with chromosomal DNA prepared from IS223
GV25	Transformation of PY436 to Cm^r with M13mp19*cat*-19 RFI DNA
GV26	As GV25, but with M13mp19*cat*-4 RFI DNA
GV27	As GV25, but with M13mp19*cat*-29 RFI DNA
GV29	As GV25, but with M13mp19*cat*-17 RFI DNA
GV50	As GV25, but with M13mp19*cat*-32 RFI DNA
GV56	Transductant of BD170 to Cm^r with SPβ lysate prepared by heat induction of Cm^r ZB307 transformants obtained with pZΔ326-GV43 DNA
GV57	As GV56, but with pZΔ326-GV49 DNA
GV58	As GV56, but with pZΔ326-GV39 DNA

and Gilbert (23) or by the method of Sanger et al. (38). Deletions into inserts in M13mp19*cat* were obtained by the method of Dale et al. (5), with oligonucleotides, enzymes, and chemical reagents purchased from International Biotechnologies, Inc., and used as instructed by the supplier.

Construction of M13mp19*cat*. A gel-purified 1-kb *Hpa*II fragment from pBD64 (10) that contained the *cat* gene was inserted by ligation into the *Ava*II site of M13mp19 after filling in the recessed ends of the *Hpa*II- and *Ava*II-generated DNAs, as described by Wartell and Reznikoff (43) (Fig. 1). Ligation products were recovered by transfection of *E. coli* JM101 (25), and insert-containing phage were identified by size after electrophoresis of single-stranded virion DNA through agarose. Because the *Ava*II site of M13mp19 is in nonessential DNA upstream from the promoter for the *lac* alpha fragment, the M13mp19*cat* construction was fully viable as a phage in *E. coli* and it produced blue plaques in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Phage growth was not noticeably impaired by the presence of the *cat* gene, nor did the *cat* gene affect the stability of inserts cloned into the phage. Moreover, all the polylinker sites in M13mp19*cat* remained unique cloning sites.

Construction of PY415 and PY436. Strain PY415 was generated by transforming strain KS181 (*spoIIE*::Tn*917* ΩHU181) to chloramphenicol resistance (Cm^r) with pTV55 DNA (47) that had been linearized by digestion with *Pst*I. This procedure replaced the transposon-associated *erm* gene with a *cat* gene and resulted in the integration of a promoterless *lacZ* gene into the chromosomal copy of Tn*917* to create a *spoIIE-lacZ* fusion. Strain PY436 was generated by transforming PY415 to macrolide-lincosamide-streptogramin B resistance (MLS^r) with pTV32 DNA (30) that had been linearized by digestion with *Pst*I. This transformation restored the transposon-associated *erm* gene and deleted the pTV55-associated *cat* gene, while leaving the *lacZ* fusion intact. The net result was equivalent to replacing the simple Tn*917* insertion in *spoIIE* with an insertion of Tn917*lac*.

Gene disruption experiments. When *B. subtilis* strains were transformed with nonreplicating plasmids or with M13 RFI DNA containing cloned chromosomal fragments, selections were always for Cm^r and always carried out with a double soft-agar overlay, as described previously (45), to allow the induction of *cat* expression before selection. With this selection protocol, we have never encountered instances of multiple integration of vectors, as reported by other investigators (33). In the present work, therefore, we have not routinely prepared Southern blots to verify the structures of integrated forms.

Integration of M13mp19*cat* constructions after generating deletions. After deletions were generated by the method of Dale et al. (5) toward or into the *spoIIE* promoter region of inserts cloned into M13mp19*cat*, RFI DNA was introduced into the chromosome of wild-type strains (see Fig. 7) or of strains containing *spoIIE*::Tn*917*ΩHU181::*lac55*::*erm32* (see Fig. 8) by integrative recombination. The selection was for Cm^r, carried out as with the gene disruption experiments described above. In the case of integrative recombination into strains containing *spoIIE*::Tn*917*ΩHU181::*lac55*::*erm32*, it was possible for integration to occur as the result of recombination between *lacZ* sequences in the chromosome and the *lac'* fragment in M13, which includes about 350 bp of *lacZ*, instead of as the result of recombination between the cloned insert and its counterpart in the chromosome. In practice, however, the desired recombination event occurred much more frequently, because the cloned insert (>1.5 kb) was significantly larger than the *lac'* fragment. Moreover, recombination between *lac* sequences

TABLE 3. Plasmids and phages used

Plasmid or phage	Source or reference
pZΔ326	30, 48
pBD64	10
pTV32	30
pTV55	47
M13mp19	44
pUC9	42
p280.1	Derived from *spoIIE*::Tn*917*ΩHU7::pTV20 (46) by digestion of chromosomal DNA with *Eco*RI, dilute ligation, and transformation into *E. coli* MM294 (24); contains 1.9 kb of genomic sequence, including the *spoIIE* promoter
p282.2	Derived from *spoIIE*::Tn*917*ΩHU7::pTV21Δ2 (46) by digestion of chromosomal DNA with *Sph*I, dilute ligation, and transformation into MM294 (24); contains 3.2 kb of genomic sequence, including the 3′ end of the *spoIIE* operon
M13mp19*cat*	This work (see Materials and Methods)
M13mp19*cat*-1	This work; subclone of a 188-bp *Msp*I-*Hin*dIII fragment containing the *spoIIE* promoter from p280.1 into the *Acc*I-*Hin*dIII backbone of M13mp19*cat*
M13mp19*cat*-4	This work; derived from chr::M13mp19*cat*-1 by partial digestion of chromosomal DNA from strain GV2 with *Hin*dIII, dilute ligation, and transfection into *E. coli* JM101 (25); contains 2.2 kb of genomic sequences, including the *spoIIE* promoter and the two *Hin*dIII fragments internal to the operon
M13mp19*cat*-17	This work; the result of a deletion into the insert in M13mp19*cat*-4 produced by the method of Dale et al. (5); deletion endpoint at −99 relative to the S1-determined start point of transcription (Fig. 5)
M13mp19*cat*-19	This work; generated as M13mp19*cat*-17; deletion endpoint at −66 relative to the S1-determined start point of transcription
M13mp19*cat*-29	This work; subclone of a 346-bp *Sau*IIIA-*Hin*dIII fragment from p280.1 containing the *spoIIE* promoter into the *Bam*HI-*Hin*dIII backbone of M13mp19*cat*
pUC9-GV47	This work; subclone of a 253-bp *Rsa*-*Hin*dIII fragment from M13mp19*cat*-29 containing the *spoIIE* promoter into the *Sma*I-*Hin*dIII backbone of pUC9
M13mp19*cat*-32	This work; subclone of the *Eco*RI-*Hin*dIII insert from pUC9-GV47 into the *Eco*RI-*Hin*dIII backbone of M13mp19*cat*
pZΔ326-GV39	This work; subclone of the *Bam*HI-*Hin*dIII insert from M13mp19*cat*-1 into the *Bam*HI-*Hin*dIII backbone of pZΔ326
pZΔ326-GV32	This work; derived from pZΔ326-GV39 by removal of the *Eco*RI site; *Eco*RI-digested pZΔ326-GV39 treated with Klenow to fill in the recessed ends and then recircularized by ligation
pZΔ326-GV34	This work; subclone of the *Bam*HI-*Hin*dIII insert from pZΔ326 into the *Bam*HI-*Hin*dIII backbone of pZΔ326-GV32; identical to pZΔ326 except for the absence of an *Eco*RI site between the *amp* gene and the *cat* gene
pZΔ326-GV43	This work; subclone of the *Eco*RI-*Hin*dIII insert from M13mp19*cat*-29 into the *Eco*RI-*Hin*dIII backbone of pZΔ326-GV34
pZΔ326-GV49	This work; subclone of the *Eco*RI-*Hin*dIII insert from pUC9-GV47 into the *Eco*RI-*Hin*dIII backbone of pZΔ326-GV34

was readily apparent, because it resulted in no *lac* fusion at all. Nevertheless, whenever M13 constructs were introduced into strains containing *spoIIE*::Tn*917*ΩHU181::*lac55*::*erm32*, five independent transformants were recovered and examined to determine whether they exhibited uniform *lac* expression.

S1 protection experiments. RNA was isolated from sporulating cultures grown in DSM broth at T_0, T_2, and T_4 by the method of Penn et al. (29). A 5′-end-labeled probe was prepared from the promoter-containing insert in pZΔ326-GV39. *Hin*dIII-cut pZΔ326-GV39 DNA was treated with alkaline phosphatase, and then end labeled by incubation with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP (6,000 Ci/mmol; Amersham Corp.). Probe was prepared by digesting labeled DNA with *Bam*HI and isolating the 201-bp promoter-containing fragment after electrophoresis through polyacrylamide. Hybridization and S1 digestion conditions were based on the procedure described by Berk and Sharp (2). Each 200 μl of hybridization reaction contained 30 μg of RNA and 250 ng of end-labeled probe. Hybridization was for 3 h at 49°C in 80% formamide–40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 6.4)]–0.4 mM NaCl–1 mM EDTA. S1 digestion was for 30 min at 45°C with 1 U/μl of S1 endonuclease (Bethesda) in 30 mM sodium acetate (pH 4.6)–20 mM NaCl–1 mM $ZnSO_4$, in the presence of 20 μg of salmon sperm DNA per ml. Treated samples were run on an 8% polyacrylamide gel with samples of the end-labeled probe subjected to the G+T or C+A reactions of Maxam and Gilbert (23).

β-Galactosidase assays. Bacteria were cultured for assay in highly aerated DSM broth at 37°C. The inoculum for such cultures was prepared by spreading a dilute suspension of a strain to be assayed over the surface of a DSM agar plate, and the plate was incubated at ambient temperature overnight. This procedure resulted in a very light lawn of growth. A suspension of bacteria was then obtained by adding 4 to 5 ml of DSM broth to the plate and rubbing with a glass rod. A portion of the suspension was used to inoculate 40 ml of DSM broth in a baffled 250-ml flask. The inoculum was adjusted such that at least 1 h of aerated incubation was required for significant turbidity to develop. In this way, even samples taken at the early stages of log-phase growth would consist uniformly of bacteria in a vegetative condition. At various intervals during growth and sporulation,

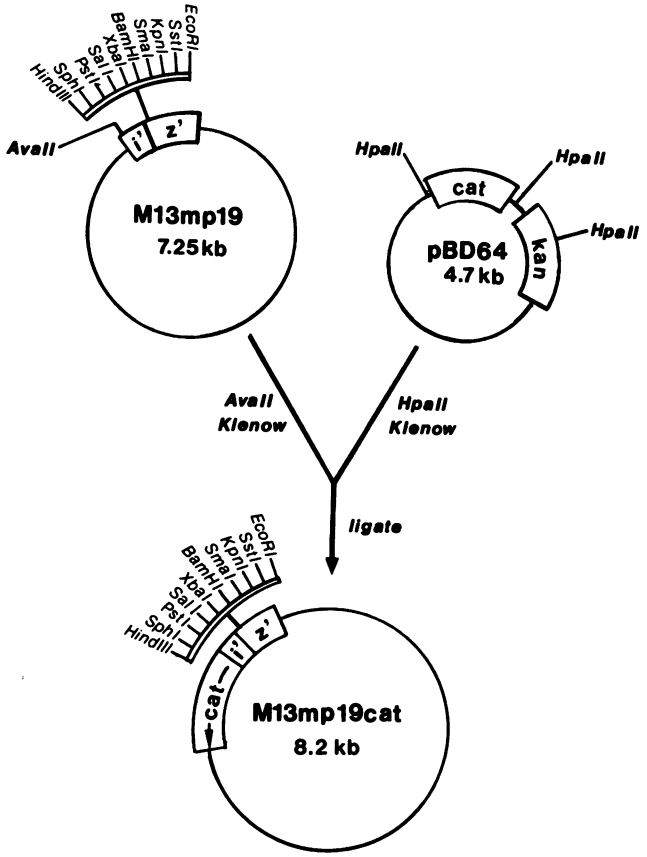

FIG. 1. Construction of M13mp19*cat*. Coliphage M13mp19 was described by Yanisch-Perron et al. (44). i', Fragment of the *E. coli* chromosome that contains the promoter for the *lac* operon; z', fragment of the *lacZ* coding sequence capable of alpha complementation (25). *B. subtilis* plasmid pBD64 was described by Gryczan et al. (10). cat, Chloramphenicol acetyltransferase gene originally associated with the *Staphylococcus aureus* plasmid pC194 (13); kan, neomycin phosphotransferase gene originally associated with the *S. aureus* plasmid pUB110. Klenow, treatment of restriction fragments with the large tryptic fragment of Pol I (16) in the presence of deoxyribonucleoside triphosphates to produce flush ends (43). Details are given in Materials and Methods. In M13mp19*cat*, the arrow associated with the *cat* gene indicates its transcriptional orientation.

0.5-ml samples were withdrawn and frozen in liquid nitrogen. β-Galactosidase activity in these samples was measured by a fluorometric assay described previously (45).

RESULTS

Use of Tn*917* insertions for initial characterization of the *spoIIE* locus. Among a collection of *spo* mutations obtained in previous work by insertional mutagenesis with Tn*917*, two mutations (*spoIIE*::Tn*917*ΩHU7 and *spoIIE*::Tn*917* ΩHU181) appeared very likely to be within the *spoIIE* locus (37). Segments of chromosomal DNA extending in either direction from these insertions were cloned in *E. coli* by using vectors pTV20 and pTV21Δ2 to produce a collection of *E. coli* plasmids containing nested sets of cloned fragments, as described previously (46). By using some of these plasmids, M. Young at the University College of Wales, United Kingdom, demonstrated that some of the cloned fragments were able to transform certain *spoIIE* recipients to Spo$^+$ (M. Young, personal communication), supporting the classification of the transposon-generated mutations as alleles of *spoIIE*.

It was apparent from the restriction maps of overlapping cloned fragments of chromosomal DNA adjacent to *spoIIE*::Tn*917*ΩHU7 and *spoIIE*::Tn*917*ΩHU181 (data not shown), together with previously published Southern blots (46), that the two transposon insertions were in opposite orientations, that they were 2.4 kb apart, and that neither insertion had been accompanied by significant deletions or other rearrangements. A physical map of the *spoIIE* region deduced from the analysis of cloned fragments is given in Fig. 2. To determine whether the two transposon insertions were within the same transcription unit and to determine how far beyond the sites of the two insertions the *spoIIE* operon might extend, the original pTV20- or pTV21Δ2-derived plasmid clones or integrational vectors containing subcloned fragments of these clones (Materials and Methods) were used in a series of gene disruption experiments. Nonreplicating plasmids carrying a selectable marker and containing cloned chromosomal fragments were used to transform a wild-type recipient strain with the expectation that transformants would result only from integration of the plasmid and would acquire a Spo$^-$ phenotype only when the cloned fragment was internal to the *spoIIE* transcription unit. The results of these experiments (Fig. 2) indicated that the two transposon insertions were in fact within the same transcription unit. Interestingly, the results also suggested that both insertions were only just within the two boundaries of the transcription unit.

To determine which boundary of the transcription unit corresponded to the promoter, a promoterless *lacZ* gene was integrated into both transposon insertions by homologous recombination. This was accomplished by transforming transposon-containing recipients to Cm^r with a linearized sample of vector pTV55 (47). A Cm^r transformant could arise only as the result of a recombination event that integrated a promoterless copy of *lacZ* into the chromosomal copy of Tn*917* in an orientation defined by the orientation of the transposon sequences themselves, and this event would result in a transcriptional gene fusion that places the expression of *lac* under the control of the *spoIIE* promoter only when the transposon is inserted such that its *erm*-proximal end is oriented toward the promoter (47). Bacteria containing *spoIIE*::Tn*917*ΩHU7 and *spoIIE*::Tn*917*ΩHU181 were transformed to Cm^r with linearized pTV55 and then transferred to DSM agar, a sporulation-inducing medium, in the presence of X-Gal. Only colonies of *spoIIE*::Tn*917*ΩHU181-containing transformants developed a blue color, suggesting that *spoIIE* was transcribed in the direction indicated in Fig. 2. To determine whether expression of a *spoIIE-lac* fusion was developmentally controlled, β-galactosidase activity of one *spoIIE*::Tn*917*ΩHU181-containing transformant (PY415) was assayed throughout growth and sporulation in DSM broth. No activity was detected until $T_{1.5}$ (Fig. 3). Relatively high levels of expression continued through at least T_{10}.

Developmental regulation of the *spoIIE* transcription unit. Because bacteria containing *spoIIE*::Tn*917*ΩHU181::*lac*-derived fusions were phenotypically Spo$^-$, we suspected that the expression patterns of these bacteria might not reflect the true pattern of *spoIIE* activity in the wild type. We were also interested in determining the effects of various stage 0 and stage II mutations on the expression of *spoIIE*. For these reasons, it was desirable to construct a *spoIIE-lacZ* fusion such that the bacteria would remain Spo$^+$. This was accomplished by subcloning a 188-bp *Msp*I-*Hin*dIII restriction fragment that we knew from additional gene disruption experiments (see below) to contain the *spoIIE* promoter into

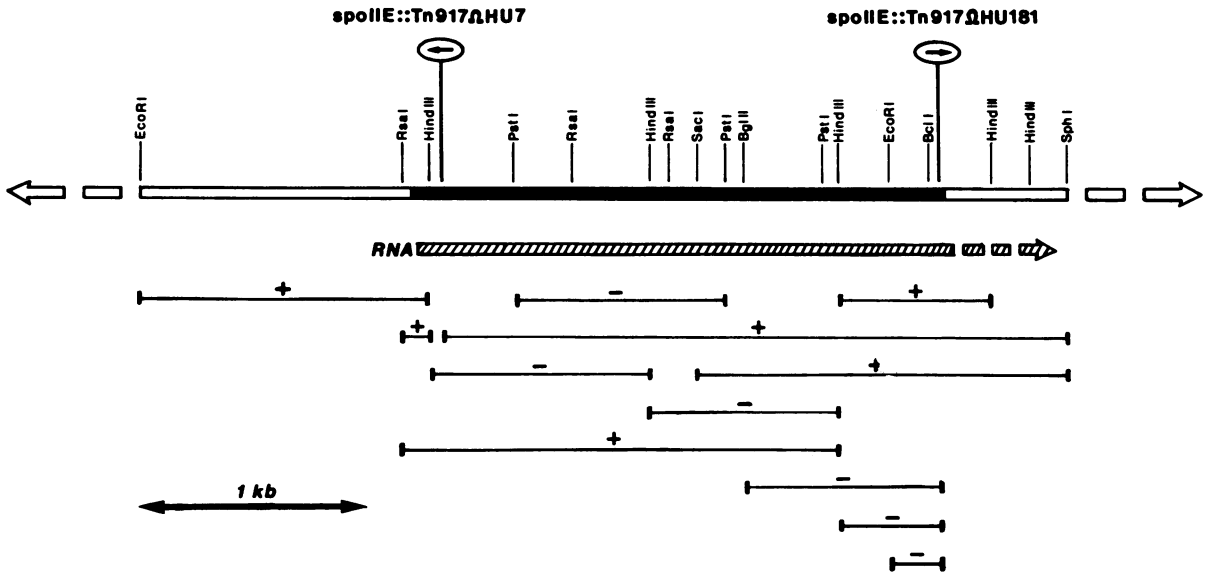

FIG. 2. Physical map of the *spoIIE* region. The positions of two Tn*917* insertional mutations, *spoIIE*::Tn*917*ΩHU7 and *spoIIE*::Tn*917*ΩHU181, are indicated relative to several mapped restriction sites. The map was compiled from the analysis of several overlapping cloned chromosomal fragments (data not shown), extending from the sites of the two transposon insertions and isolated with the use of pTV20 and pTV21 (46). The solid area represents the region defined by gene disruption studies as being within the functional boundaries of the *spoIIE* operon. Subcloned genomic fragments used in the gene disruption experiments are indicated at the bottom. +, Spo$^+$ transformant produced when the indicated fragment was transferred to an integrational vector (M13mp19*cat*) and forced into the chromosome by integrative recombination; −, Spo$^-$ transformant produced. The positions of restriction sites for *Pst*I, *Rsa*I, *Sac*I, *Bgl*II, and *Bcl*I were not determined in regions external to the solid area. The positions of *Eco*RI and *Sph*I sites relative to *spoIIE*::Tn*917*ΩHU7 were confirmed in previous work by Southern blots (46).

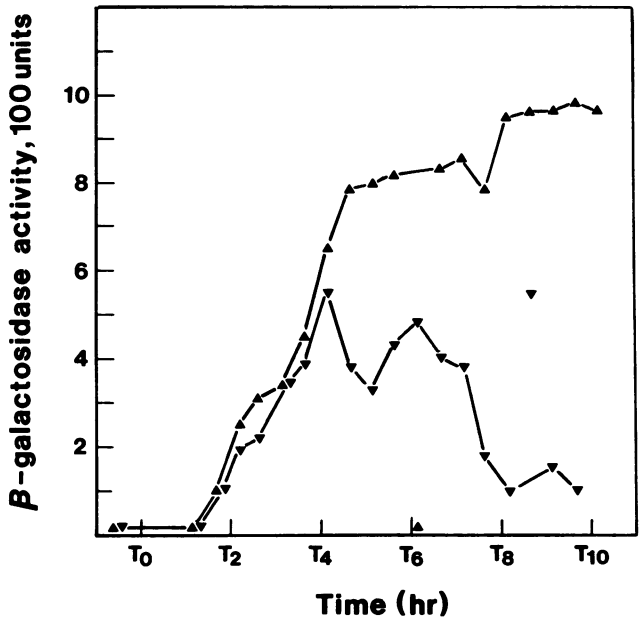

FIG. 3. Expression of *spoIIE-lacZ* transcription gene fusions. Strains PY415 (▲) and GV1 (▼) were cultured in DSM broth as described in Materials and Methods. Growth was monitored by measuring culture turbidity, and T_0 marks the end of log-phase growth. Culture samples were assayed for β-galactosidase activity as described by Youngman (45).

pZΔ326 (30, 48), an integrational vector with cloning sites just upstream of a promoterless *lacZ* gene. Integration of this construction into the chromosome of a wild-type strain (BD170) yielded a Spo$^+$ transformant (GV1) that exhibited the *lac* expression pattern shown in Fig. 3. The Spo$^+$ pZΔ326-derived fusions and the Spo$^-$ *spoIIE*::Tn*917*Ω HU181::*lac*-derived fusions became active at the same time but differed markedly in the persistence of activity at later times. This difference was subsequently confirmed in an isogenic background to be produced by the *spoIIE* mutation itself (data not shown).

To determine how *spo* mutations that block sporulation at an early stage might affect the expression of *spoIIE*, an isogenic set of strains was constructed containing the pZΔ326-derived fusion in combination with mutant alleles of various stage 0 and stage II loci. These strains were assayed for *lac* expression throughout growth and sporulation (Fig. 4). Alleles of all stage 0 loci, except *spo0J*, blocked expression completely; *spo0J* mutants displayed abut 20% of the wild-type activity. In all stage II mutants tested, activity was either unimpaired or was actually somewhat enhanced.

High-resolution S1 protection analysis of the *spoIIE* promoter region. As discussed above, results of gene disruption experiments had indicated that the *spoIIE* promoter was within a 188-bp *Msp*I-*Hin*dIII restriction fragment. The complete DNA sequence of this fragment plus an additional 65 bp that extends upstream to an *Rsa*I site is shown in Fig. 5. To determine the location of transcription start point(s) in the promoter region, an S1 protection experiment was carried out by using as the 5′-end-labeled protection probe an *Msp*I-*Hin*dIII fragment that had been subcloned into M13mp19 (44). As a result of this subcloning, 13 bp of polylinker DNA became appended to the 3′ end of the protection probe. The results of this experiment, which used

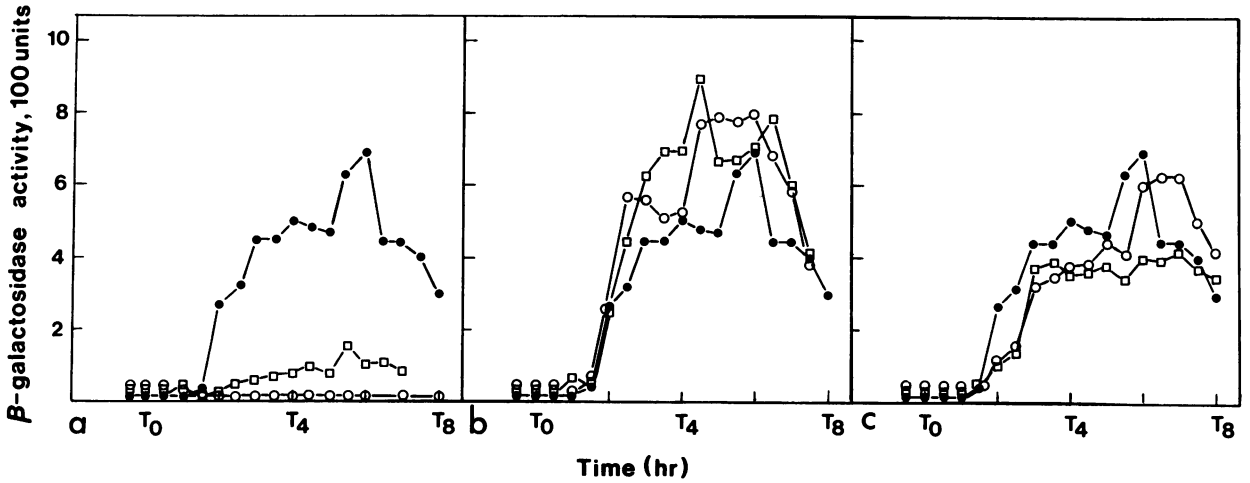

FIG. 4. Effects of stage 0 and stage II mutations on the expression of *spoIIE-lacZ* transcriptional fusions. Strains were cultured and assayed as described in the legend to Fig. 3. (a) ●, GV1, a Spo$^+$ strain; □, GV3 (*spo0J93*); and ○, GV15 (*spo0C9V*), GV16 (*spo0F221*), GV18 (*spo0E11*), GV19 (*spo0A12*), GV22 (*spo0B136*), and GV23 (*spo0HΔHind*), whose activities were indistinguishable from that of a control strain that did not contain a fusion (result not shown). (b) ●, GV1; ○, GV4 (*spoIIAC*); and □, GV11 (*spoIIG55*). (c) ●, GV1; ○, GV13 (*spoIIB131*); and □, GV17 (*spoIID66*).

RNA isolated from culture samples at T_0, T_2, and T_4, are shown in Fig. 6. With the RNA isolated at T_2, a single primary band set was detected approximately 70 bp upstream from the *Hin*dIII site. The most prominent band of the set corresponds to the adenine residue indicated in Fig. 5 as +1. A much weaker signal at the same position was detected with RNA isolated at T_4, and no detectable quantity of probe was protected with T_0 RNA. In addition, no protected fragment 13 bp smaller than the full-length probe was observed with T_2 RNA, indicating that no significant initiation of transcription occurs upstream of the *Msp*I site.

Several features of the DNA sequence shown in Fig. 5 should be noted. First, at positions −10 and −35, there are no sequences resembling the RNA polymerase-binding sites utilized by any of the holoenzyme forms thus far characterized in *B. subtilis* (6). Interestingly, however, centered at −17 is the TATAAT sequence, which is a perfect match to the −10 consensus sequence for the σ^A (σ^{43})-associated holoenzyme, and centered at −43 is the TTGACA sequence, which is a perfect match to the −35 consensus sequence for the same holoenzyme form. Although there is nothing resembling the AT box present upstream of the *spoVG* promoter (27), there are prominent runs of T residues. Moreover, repeated at three positions within the interval from −47 to −112, a region demonstrated below to regulate the expression of the *spoIIE* promoter, these T residues are part of a 7-bp sequence motif, TTCTTTT. Finally, centered at −155 is an obvious sequence of hyphenated dyad symmetry followed by a run of T residues, which would be expected to function as a factor-independent terminator of transcription (35).

Deletion analysis of the *spoIIE* promoter region by using

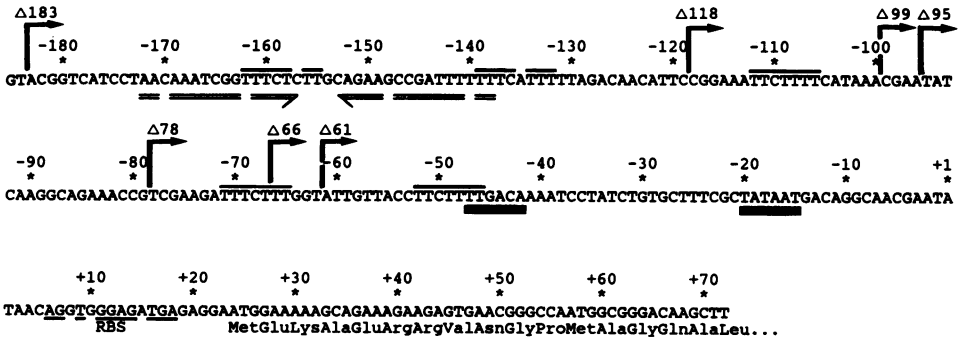

FIG. 5. DNA sequence of the *spoIIE* promoter region. +1, Apparent start point of transcription as determined by the S1 protection results (see Fig. 6). Heavy bars under the sequence indicate sequences that match the −10 and −35 consensus sequences for RNA polymerase associated with σ^A (σ^{43}); light bars labeled RBS under the sequence indicate nucleotides that are presumed to form a ribosome-binding site and nucleotide residues that would pair with the 3′ end of the 16S rRNA of *B. subtilis* (28). Just downstream from RBS, at the appropriate spacing, is a methionine codon that begins an open reading frame of over 300 codons (P. Guzmán and J. Westpheling, unpublished data). Light bars over the sequence indicate sequences related to the TTCTTTT motif, the significance of which is unknown. Also indicated are the endpoints of deletions whose effects on *spoIIE* promoter activity are summarized in Table 4. The endpoints of three of the deletions correspond with restriction sites: deletion Δ183 was produced by truncation with *Rsa*I, deletion Δ118 was produced by truncation with *Msp*I, and deletion Δ78 was produced by truncation with *Taq*I. Finally, double underscoring with arrowheads indicates the location of a sequence of hyphenated diad symmetry, whose function may be to terminate transcription toward the promoter from an adjacent transcription unit 5′ to the *spoIIE* operon.

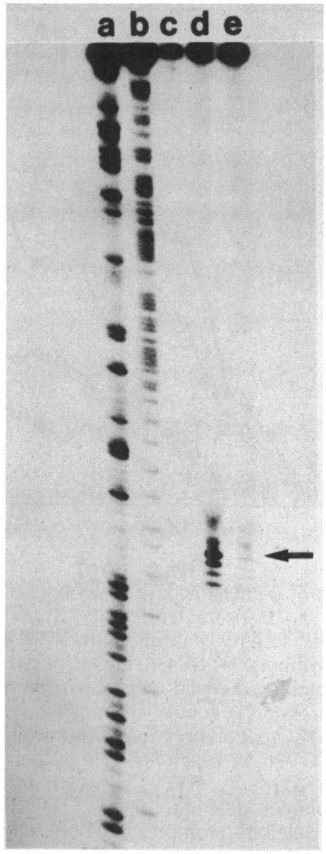

FIG. 6. Results of a high-resolution S1 protection experiment to determine the start point of transcription from the *spoIIE* promoter. RNA isolated from a sporulating culture of strain BD170 at T_0 (lane c), T_2 (lane d), or T_4 (lane e) was hybridized with a single-stranded DNA probe end labeled at the *Hin*dIII site centered at +70 in the sequence shown in Fig. 5. After treatment with S1 endonuclease (Materials and Methods), samples were denatured and run on an 8% polyacrylamide gel along with samples of the same probe subjected to the G+T (lane a) or C+A (lane b) sequencing reactions of Maxam and Gilbert (23). Arrow, Position of the protected probe.

M13mp19*cat*. To identify and delimit sequences important for the regulation of *spoIIE* promoter activity, a series of deletions was generated toward the transcription start site from the upstream direction. Our approach for generating these deletions was to use a novel integrational vector referred to as M13mp19*cat* (Fig. 1). This vector was derived from M13mp19 (44) by the insertion of a *cat* gene that confers Cm^r in *B. subtilis* at an *Ava*II site just upstream of the *lacZ'* promoter. M13mp19*cat* thus remained viable as a phage in *E. coli* that can make blue plaques in the presence of X-Gal unless foreign DNA is inserted into one of the polylinker sites. When the foreign DNA is a fragment of the *B. subtilis* chromosome, the double-stranded RFI form of the insert-containing vector can be introduced by transformation into a *B. subtilis* recipient, and the DNA will integrate into the chromosome by homologous recombination, the *cat* gene affording a selection to recover recombinants. A preliminary description of the construction and properties of M13mp19*cat* was published previously (11).

The strategy for generating deletions involved cloning promoter-containing restriction fragments into polylinker sites of M13mp19*cat* in orientations that placed the polylinker *Eco*RI site upstream of the transcription start site. Deletions from the upstream direction were then generated by the method of Dale et al. (5), by which a 22-base oligomer complementary to a portion of the polylinker was annealed to the single-stranded form of the insert-containing phage, making it possible to cleave at the *Eco*RI site. A series of unidirectional deletions into the cloned insert was produced by exposing the linearized single-stranded phage to the 3'-to-5' exonuclease activity of T4 DNA polymerase for various lengths of time. Treated molecules were then recircularized as described by Dale et al. (5) and recovered upon transfection of an *E. coli* strain as phage plaques.

One advantage of this approach was that deletions could be sequenced and their effects on transcription could be determined without additional subcloning. Cultures prepared from single plaques of deletion-bearing phage were the source of both single-stranded virion DNA for sequencing and double-stranded RFI DNA for transformation into *B. subtilis*. The endpoints of deletions characterized in detail are indicated in Fig. 5. Two kinds of transformation exper-

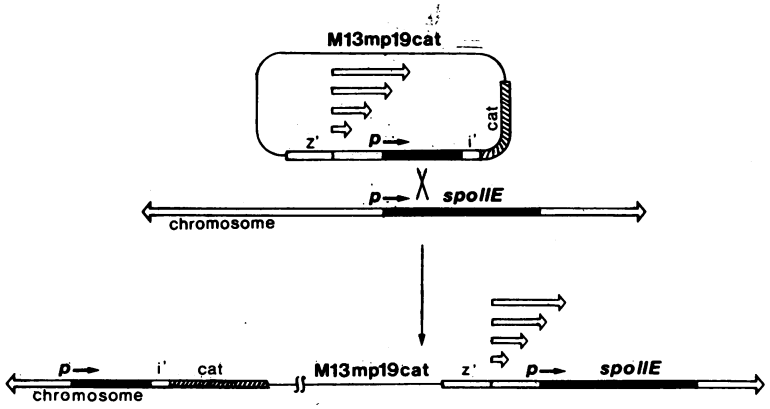

FIG. 7. Introduction of deleted promoters into the *B. subtilis* chromosome by integrative recombination. Solid areas represent fragments or complete copies of the *spoIIE* operon, and p → represents the promoter for the operon. Other features of M13mp19*cat* are as described in the legend to Fig. 1. The diagram shows double-stranded RFI DNA of an M13mp19*cat* clone carrying a restriction fragment containing the promoter for the *spoIIE* operon being introduced into a wild-type *B. subtilis* strain by transformation. Deletions toward the promoter (open arrows) had been generated from the 5' direction by the method of Dale *et al.* (5). As illustrated, integrative recombination places the expression of the intact copy of the *spoIIE* operon present in the chromosome of the transformation recipient under the control of the deleted promoters. The figure is reproduced from reference 11 with permission from the publisher.

TABLE 4. Effects of deletions on *spoIIE* promoter activity

Deletion[a]	β-Galactosidase activity (% of wild type)[b]	Sporulation[c]
Δ276	100	+
Δ183	100	+
Δ118	50	+
Δ99	20	+
Δ95	ND	+
Δ78	ND	−
Δ66	<1	−
Δ61	ND	−

[a] Derivations of deletions are described in the legend to Fig. 9.
[b] Determined at T_3, as described in the legend to Fig. 3.
[c] Qualitative assessment based on colony morphology and microscopic examination.

iments with RFI DNAs were carried out to assess the effects of these deletions on promoter activity. To determine whether deletions impaired promoter activity sufficiently to produce a Spo⁻ phenotype, RF DNAs were simply transformed into a wild-type recipient with a selection for Cm^r. The consequence of integrative recombination of RF DNAs, in this case, was to place expression of the intact *spoIIE* operon in the chromosome of the recipient under control of the deleted promoters introduced by transformation (Fig. 7 and Table 4). Deletions with endpoints at −99 and −95 retained enough promoter activity to permit apparently normal sporulation, but deletions with endpoints at −78 and −67 did not. To evaluate quantitatively the effects of these deletions on promoter function, RF DNAs were transformed into a recipient that contained a *spoIIE-lacZ* fusion derived from *spoIIE*::Tn*917*ΩHU181 (Materials and Methods). In this case, integrative recombination of RF DNAs placed the expression of transposon-associated *lac* fusions under the control of deleted promoters (Fig. 8 and 9 and Table 4). As expected, deletions with endpoints at −183 had no effect, and deletions with endpoints at −67 eliminated all detectable transcription. Interestingly, however, although deletions with endpoints as far downstream as −95 had not detectably impaired sporulation, which might naively be interpreted as indicating no effect on promoter activity, deletions with endpoints as far upstream as −118 reduced the activity of a *spoIIE-lacZ* transcriptional fusion to less than 50% of that of

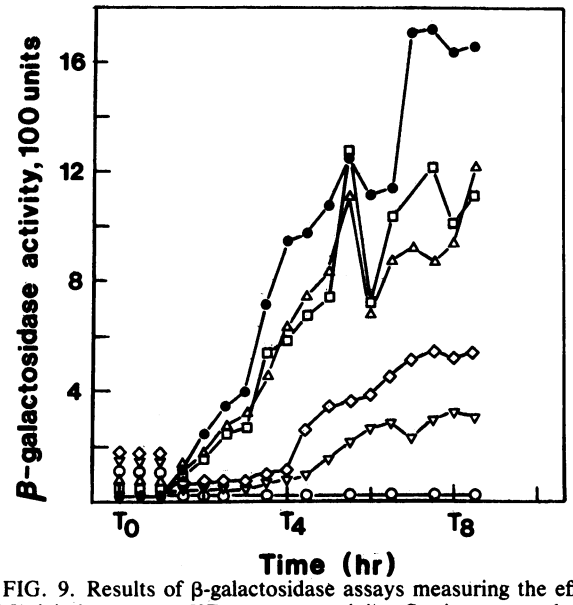

FIG. 9. Results of β-galactosidase assays measuring the effects of 5′ deletions on *spoIIE* promoter activity. Strains were cultured, and β-galactosidase activity was determined as described in the legend to Fig. 3. Symbols: ●, PY436, a control strain in which *lacZ* expression is directed by the wild-type *spoIIE* promoter; □, GV27, in which *lacZ* expression is controlled by promoter deletion Δ276; △, GV50, in which *lacZ* expression is controlled by promoter deletion Δ183; ◇, GV26, in which *lacZ* expression is controlled by promoter deletion Δ118; ▽, GV29, in which *lacZ* expression is controlled by promoter deletion Δ99; ○, GV25, in which *lacZ* expression is controlled by promoter deletion Δ66.

wild type; deletions with endpoints at −95 reduced activity to 20%. Thus, an interval of DNA extending at least 67 bp upstream from the start point of transcription is absolutely essential for the activity of the *spoIIE* promoter, and sequences extending more than 120 bp upstream are required for expression at the wild-type level. Moreover, the expression of *spoIIE* gene products at less than 20% of the wild-type level would appear to be adequate for qualitatively normal sporulation, at least under the conditions tested.

Activity of SPβ-borne *spoIIE-lacZ* fusions. To examine in Spo⁺ bacteria the activity of *spoIIE-lacZ* fusions driven by

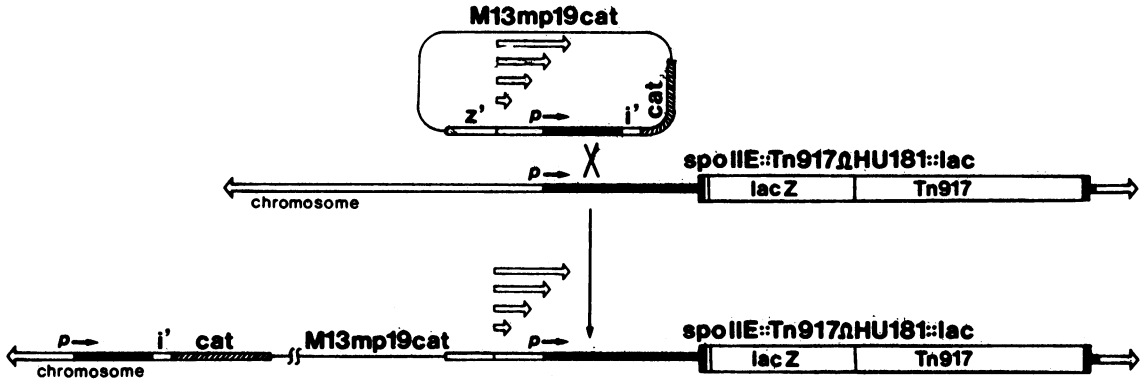

FIG. 8. Formation by recombination of *lacZ* transcriptional fusions to quantitate the effects of promoter deletions. Specific features of the represented structures are as described in the legends to Fig. 1 and 7. As with Fig. 7, the figure shows double-stranded RFI DNA of an M13mp19*cat* clone carrying a restriction fragment containing the *spoIIE* promoter being introduced by transformation into a *B. subtilis* recipient. Here, however, the recipient already contains a *spoIIE-lacZ* transcriptional fusion mediated by Tn*917*. As illustrated, integrative recombination places the expression of the fusion under the control of the deleted promoters. The figure is reproduced from reference 11 with permission from the publisher.

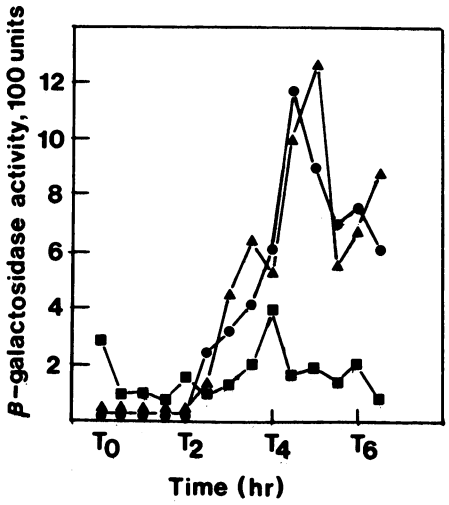

FIG. 10. Results of β-galactosidase assays monitoring the expression of SPβ-borne *spoIIE-lacZ* fusions. Strains were cultured and β-galactosidase activity was determined as described in the legend to Fig. 3. Symbols: ●, GV56, in which *lacZ* expression is controlled by a *spoIIE* promoter including 276 bp of *spoIIE* DNA 5′ to the start point of transcription; ▲, GV57, in which *lacZ* expression is controlled by a *spoIIE* promoter including 183 bp of 5′ sequence; ■, GV58, in which *lacZ* expression is controlled by a *spoIIE* promoter including 118 bp of 5′ sequence.

deleted derivatives of the *spoIIE* promoter, partial diploids were constructed in which the fusions were integrated into the prophage of the *B. subtilis* temperate phage SPβ. This was accomplished by first inserting restriction fragments containing the truncated *spoIIE* promoters into pZΔ326 (30, 48) to produce a set of plasmid-borne *spoIIE-lacZ* fusions. These fusions could then be transferred to the SPβ prophage by homologous recombination, as described by Zuber and Losick (49). Consistent with the information derived from the fusion studies discussed above, the results (Fig. 10) show that fusions driven by promoter-containing fragments that included 183 and 276 bp of DNA upstream from the start point of transcription displayed activity comparable with that of the wild type both in timing and in level of expression, but fusions driven by fragments containing 118 bp of upstream sequence displayed significantly less activity. Phage-borne fusions driven by promoters truncated to −118 also displayed some *lac* expression during the vegetative phase (Fig. 10). This result was also observed with other constructions in which truncations were more extreme and in which the *spoIIE* promoter was apparently inactive (data not shown). This finding suggests that vegetative expression is simply a background of nonspecific transcription probably originating in vector sequences. This nonspecific transcription is presumably not detected in constructions that contain more upstream DNA because of the terminatorlike sequence present in the interval between −130 and −170.

DISCUSSION

The *spoIIE* locus of *B. subtilis* consisted of a single, continuous transcription unit about 2.5 kb in size. The expression of this transcription unit was driven by a single, developmentally regulated promoter that became active during the second hour of sporulation. Activation of the *spoIIE* promoter was prevented in all stage 0 mutants (although a *spo0J* mutation [*spo0J93*] was only partially inhibitory) but was essentially unaffected by mutations in all stage II loci tested.

The failure of mutations in *spoIIA* and *spoIIG* to affect the expression of *spoIIE* is of particular interest. The *spoIIA* and *spoIIG* operons encode RNA polymerase σ factors or σ-like proteins that presumably appear in their active forms at about 90 min after the start of sporulation, or at approximately the time when the expression of *spoIIE* is induced. In the case of *spoIIG*, one of the encoded gene products is σ^E (σ^{29}), a protein which functions as a sporulation-specific σ factor (40). Although the *spoIIG* operon itself is induced at T_0 (15), σ^E appears first as an inactive precursor which is not processed into its active form until the second hour of sporulation (17, 41). In the case of *spoIIA*, an operon which is not transcriptionally active until the second hour of sporulation (8, 31), the deduced amino acid sequence of one of the open reading frames, *spoIIAC*, exhibits strong similarity to other known σ factors (7). The *spoIIAC* gene product has not actually been demonstrated to function as a polymerase σ factor, however. Because the expression of *spoIIE* is independent of *spoIIA* and *spoIIG*, the transcriptional activation of *spoIIE* cannot be the result of the appearance of known σ factors or σ-like proteins; activation must involve either the appearance of another yet-to-be-discovered σ factor or must occur by some entirely different mechanism that makes use of holoenzyme forms associated with σ^A (σ^{43}), σ^B (σ^{37}), σ^C (σ^{32}), σ^D (σ^{28}), or σ^H (σ^{30}), holoenzyme forms thus far implicated only in the expression of vegetative genes or genes activated at T_0 (20).

The functional boundaries of the *spoIIE* promoter were investigated by generating a series of deletions toward the promoter from the 5′ direction. The activity of promoters suffering these deletions was assessed qualitatively by observing the effects on sporulation when these promoters were driving the expression of the *spoIIE* operon and quantitatively by measuring β-galactosidase produced when these promoters were driving the expression of a *lacZ* transcriptional fusion. The end points of deletions were established by DNA sequencing relative to a presumptive in vivo start point of transcription which had been determined by high-resolution S1 protection mapping. Interestingly, deletions quite far upstream from this start point reduced transcription significantly. An interval of DNA extending more than 120 bp upstream is apparently required for full activity of the promoter. Deletions toward the promoter from −118 progressively reduced transcription until all detectable transcription was eliminated at −67. We conclude from this finding that one element of the transcriptional control of *spoIIE* may be an absolute requirement for a positive regulatory factor that interacts with sequences upstream from −67. We note also the presence of a sequence motif, TTCTTTT, repeated at three positions between −47 and −112, which could be specific sites of interaction for such a factor.

The DNA interval between −67 and +1 was also examined for sequences resembling the −35 and −10 consensus sequences recognized by characterized forms of *B. subtilis* RNA polymerase. We detected the sequence TATAAT, centered at −17, which was a perfect match to the −10 consensus sequence for $E\sigma^A$ (σ^A-associated holoenzyme), and centered at −44 was the sequence TTGACA, which was a perfect match to the −35 consensus sequence for $E\sigma^A$. The spacing between these consensus sequences was 21 bp, however. The typical spacing in a conventional promoter recognized by $E\sigma^A$ is 17 to 19 bp, and consensus sequences spaced 21 bp apart would not be expected to constitute a

FIG. 11. Comparison of the putative RNA polymerase-binding regions of the *spoIIE* and *spoIIG* promoters. The *spoIIG* sequence was taken from Kenney and Moran (15).

functional Eσ^A promoter (28). Thus, not only are the σ^A-like −10 and −35 consensus sequences in the *spoIIE* promoter inappropriately positioned relative to the apparent start point of transcription, but they are also inappropriately positioned relative to each other. The former observation might be explained as reflecting a degradation or processing artifact in the S1 protection experiments that determined the transcription start point, but the latter observation is more difficult to dismiss. Nevertheless, although we regard as an open question whether the *spoIIE* promoter is actually utilized by Eσ^A, we consider it very likely that the σ^A-like −10 and −35 consensus sequences within the promoter function as polymerase recognition elements. The basis for this hypothesis is the fact that similarly spaced σ^A-like −10 and −35 consensus sequences are also present in another developmentally regulated promoter, the promoter for the *spoIIG* operon. When we aligned our sequence of the *spoIIE* promoter with a sequence of the *spoIIG* promoter published recently by Kenney and Moran (15) (Fig. 11), the spacing between the σ^A-like consensus sequences in *spoIIG* was 22 bp. We consider it unlikely that this unusual configuration of consensus sequences would be present in these two promoters by chance.

How might the inappropriately spaced σ^A-like consensus sequences in the *spoIIE* and *spoIIG* promoters function as RNA polymerase-binding sites? Sequences with spacings of 21 to 22 bp are too far apart by one-half turn of the helix to form a promoter that could be recognized by the conventional form of Eσ^A. One possibility is that the inappropriately spaced consensus sequences can interact with a modified form of Eσ^A. Another possibility is that with the assistance of a helix-distorting protein that may bind to the promoter region, the consensus sequences might be able to interact with the normal form of Eσ^A. Or perhaps, the *spoIIE* and *spoIIG* promoters are recognized by a novel holoenzyme form associated with a σ factor distinct from σ^A, which shares specificity for the same consensus sequences but which demands a different spacing of these sequences.

It is interesting that the *spoIIE* and *spoIIG* operons are quite different in their temporal regulation: the former became transcriptionally active at $T_{1.5}$ (this work), while the latter is induced at T_0 (15). Thus, if the promoters of these operons are recognized by the same form of RNA polymerase, temporal regulation must be accomplished by a mechanism different from activation in response to the appearance of a particular σ species. Consistent with this hypothesis is the fact that although the putative polymerase-binding regions of *spoIIE* and *spoIIG* displayed significant similarities even apart from the −10 and −35 consensus sequences (Fig. 11), *spoIIE* and *spoIIG* were completely divergent in sequences upstream of the binding region (not shown in the case of *spoIIG*) over an interval that we know is essential for transcriptional activity (at least in the case of *spoIIE*).

Integrational vectors are particularly useful in *B. subtilis*, in part because of the very efficient and highly recombinogenic nature of DNA uptake by these bacteria in the state of natural transformation competence. Vectors in conventional use consist of ColE1-derived replicons that carry a drug resistance marker selectable in single copy in a *B. subtilis* host. We have accomplished in this work the construction and use of a novel kind of integrational vector based instead on the single-stranded filamentous coliphage M13. The double-stranded RFI form of these vectors features all the advantages associated with conventional plasmid-derived vectors, whereas the single-stranded virion DNA facilitates additional manipulations useful for delimiting and analyzing promoter-associated sequences that regulate promoter activity. The specific application we have demonstrated in this work is the use of these vectors to generate unidirectional deletions into promoter-containing cloned inserts by the method of Dale et al. (5). The advantage of this approach consists, first, in the ease with which a collection of deletions can be generated (unlike *Bal* 31- or exonuclease III-generated resections, deleted molecules can be readily recircularized without treatments to produce flush ends, linker attachment, or subcloning), second, in the ease with which the deletions can be characterized (they can be sequenced directly by using the M13 universal primer), and third, in the ease with which the effects of the deletions on promoter function can be evaluated (RFI DNA can be introduced by Campbell recombination directly into the chromosome of a wild-type strain or a strain containing a transcriptional fusion to the gene under study). In addition, we anticipate that these vectors will facilitate other manipulations for which the availability of cloned promoter-containing fragments in a single-stranded form is desirable, such as bisulfite-mediated or oligonucleotide-mediated mutagenesis.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant GM35495 from the National Institutes of Health.

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