JOURNAL OF BACTERIOLOGY, Feb. 1989, p. 692–698
0021-9193/89/020692-07$02.00/0

Vol. 171, No. 2

Regulation of Transcription of the *Bacillus subtilis spoIIA* Locus

JIUNN-JONG WU, MARY GAUKLER HOWARD, AND PATRICK J. PIGGOT*

Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Received 15 September 1988/Accepted 24 October 1988

The start point of *spoIIA* transcription was defined by primer extension analysis with two separate primers. It was 27 bases upstream from the putative translation initiation codon of the first open reading frame in the *spoIIA* locus. A region extending at least 52 bases upstream from the transcription start site was necessary for transcription, as determined with integrative plasmids. Transcription of *spoIIA* was dependent on the *spo0A*, *spo0B*, and *spo0F* loci, but this dependency was partly overcome by increasing the number of copies of the *spoIIA* promoter region. Transcription of *spoIIA* was absolutely dependent on the *spo0H* locus, which codes for the RNA polymerase sigma factor σ^H. Regions approximately −35 and −10 upstream from the *spoIIA* transcription start site showed sequence homology with *Bacillus subtilis* σ^H promoters.

Spore formation by *Bacillus subtilis* is a system of cellular differentiation. The system is primitive; nevertheless, it requires the expression of more than 50 sporulation-specific genetic loci (21). A number of the sporulation-specific *spo* loci (defined by mutations that block sporulation) are likely to have regulatory roles, and this is fairly certainly the case with those loci that code for RNA polymerase sigma factors (4, 35). The circumstantial evidence is now very good that *spoIIA* belongs to this group of regulatory loci. We have been studying how expression of *spoIIA* is itself regulated.

The *spoIIA* locus was defined by a group of tightly linked mutations that blocked sporulation at septum formation (17, 25, 27). The locus is an operon containing three open reading frames transcribed as a single polycistronic message (11, 29, 33). The protein product, SpoIIAC, of the third open reading frame bears extensive sequence homology with RNA polymerase sigma factors (5, 15) and has recently been isolated and shown to confer transcriptional specificity to core RNA polymerase in vitro (P. Setlow, personal communication). The nature of SpoIIAA and SpoIIAB is unknown, although it is possible that SpoIIAA acts synergistically with SpoIIAC (21). Transcription of *spoIIA* commences about 60 min after the start of sporulation (6, 26, 28, 33, 34). (It is not possible to make exact comparisons, because estimates of the start time for sporulation are imprecise in most systems.) Evidence has been adduced that *spoIIA* is under positive control (28). In this paper we identify the transcription start point and delimit the 5′ *spoIIA* control region. We investigate the roles of the *spo0* loci in controlling *spoIIA* expression.

MATERIALS AND METHODS

Bacterial strains. The *Escherichia coli* strain used, DH5α [F^- *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* λ^- *recA1 gyrA96 relA1* Δ(*lacZYA-argF*)U169 ϕ80d*lacZ*ΔM15], was kindly provided by B. Moldover. The *B. subtilis* 168 strains used are listed in Table 1. The JH series strains were kindly provided by J. A. Hoch (24) and were isogenic with each other. The SL series strains are isogenic with MB24 except for the markers shown; they are less closely related to the JH series strains. *B. subtilis* strains containing a single copy of a plasmid integrated into the chromosome were constructed by transformation with the plasmid as a donor and were maintained on media containing 3 to 5 μg of chloramphenicol per ml. Strains with multiple copies of an integrated plasmid were maintained on media containing a constant level (which could be between 20 and 80 μg/ml) of chloramphenicol for at least three subcultures before they were used. In all strains containing integrated plasmids, the structure of the integrated plasmid was confirmed by Southern hybridizations of appropriately restricted DNA.

Plasmids. All plasmids were maintained in *E. coli* DH5α unless otherwise stated. Plasmid pHM2 was described previously (20); it contains the first two open reading frames of the *spoIIA* locus together with the *B. subtilis* region extending about 2 kilobases (kb) upstream from *spoIIA* (Fig. 1; the full extent of the insert of pHM2 is not shown). Plasmid pJM783 was a gift of J. A. Hoch and has been described by Ferrari et al. (8); it is unable to replicate in *B. subtilis* but has a chloramphenicol resistance determinant that can be expressed in *B. subtilis*. Plasmid pPP51 was constructed by cloning a 2.2-kb *Hin*dIII-*Sal*I fragment from pHM2 into pJH101 (9) that had been cut with *Hin*dIII and *Sal*I; it includes all of *spoIIAA* and *spoIIAB* (the cloned fragment included 276 base pairs of vector DNA as well as DNA from the *spoIIA* region). Plasmid pPP81 contains a *spoIIA-lacZ* transcriptional fusion and was described previously (28). Plasmid pPP157 contains a 1.5-kb *Hin*dIII-*Pvu*II fragment from pPP51 cloned into pUC18 cut with *Hin*dIII and *Sma*I. A 690-base-pair *Spe*I-*Bgl*II fragment from pPP51 was treated with DNA polymerase Klenow fragment to generate blunt ends and ligated into *Sma*I-digested pJM783 to construct pPP161. Plasmid pPP164 was constructed by replacing the small *Sma*I-*Bam*HI fragment in pJM783 with an 873-base-pair *Ssp*I-*Bgl*II fragment from pPP51. Plasmid pPP165 was constructed by replacing the small *Sma*I-*Bam*HI fragment in pJM783 with an 808-base-pair *Sca*I-*Bgl*II fragment from pPP51. The *B. subtilis* regions in the plasmids are shown in Fig. 1. All constructions were checked by analyzing digestion patterns obtained with appropriate endonucleases.

DNA preparation. Plasmid DNA was prepared from *E. coli* by the method of Guerry et al. (12) or, for small-scale preparation, by the method of Ish-Horowicz and Burke (18).

High-molecular-weight *B. subtilis* DNA susceptible to restriction endonucleases was prepared by using agarose beads as described previously (29).

DNA sequencing. DNA sequencing was by the dideoxy-chain termination method of Sanger et al. (32). A Sequenase reagent kit (United States Biochemical Corp., Cleveland, Ohio) was used according to the protocol of the manufac-

* Corresponding author.

TABLE 1. *B. subtilis* strains used

Strain	Genotype
JH646	*trpC2 phe-1 spo0A12*
JH703*abr4*	*trpC2 phe-1 spo0A677 abrB4*
MB24	*trpC2 metC3 rif-2*
SL513	*trpC2 spo0H17 rif-2*
SL566	*phe-12 spo0A34 rif-2 tal-1*
SL964	*metC3 spo0B136 tal-1*
SL965	*metC3 spo0F221 tal-1*

turer. The DNA to be sequenced was purified as a covalently closed plasmid by CsCl gradient centrifugation.

Two oligonucleotide primers were synthesized with an Applied Biosystems model 380B DNA synthesizer and kindly provided to us by J. K. de Riel. They were designed to be complementary to mRNA for *spoIIAA*. The first primer, 5′ CCAAGCCCCGAGCTGTCC 3′, is an 18-mer corresponding to bases 553 to 570 in the published sequence (11) and is 195 to 212 bases downstream from the transcription start point deduced in this paper. The second primer, 5′ GGTGATCGAGTTCGCC 3′, is a 16-mer corresponding to bases 443 to 458 in the published sequence (11) and is 85 to 100 bases downstream from the transcription start.

RNA preparation. Bacteria were grown in modified Schaeffer sporulation medium at 37°C. Separate 30-ml cultures were harvested at the end of exponential growth and at hourly intervals thereafter, and RNA was extracted by the method of Penn et al. (23) with modifications in addition to those of Zuber et al. (37).

Bacteria were harvested by centrifugation and suspended in 2 ml of LETS buffer (0.1 M LiCl, 10 mM EDTA, 10 mM Tris hydrochloride [pH 7.4], 1% sodium dodecyl sulfate). To this was added 2 ml of phenol-chloroform-isoamyl alcohol (25:24:1) and 1 ml of washed glass beads (0.45 to 0.50 mm, Glasperlen; B. Braun Melsungen, Fisher Scientific Co.). The mixture was vortexed for 4 min and then centrifuged for 10 min. The upper phase was collected, and the extraction with phenol-chloroform-isoamyl alcohol was repeated three times. LiCl to 0.2 M was added to the aqueous phase, followed by 2 volumes of alcohol. The mixture was chilled with alcohol-dry ice. After 30 min the pellet, containing the RNA, was harvested by centrifugation, suspended in 0.5 ml of diethylpyrocarbonate-treated water, and extracted twice with phenol-chloroform-isoamyl alcohol. Potassium acetate was added to 0.3 M in the aqueous phase, followed by 2 volumes of ethanol. The RNA-containing pellet was collected by centrifugation, washed twice with 70% (vol/vol) ethanol and then dried. The pellet was then dissolved in 100 μl of diethylpyrocarbonate-treated water and frozen at −70°C. The RNA was analyzed by electrophoresis to test for the quality of rRNA and by measuring A_{260} and A_{280}.

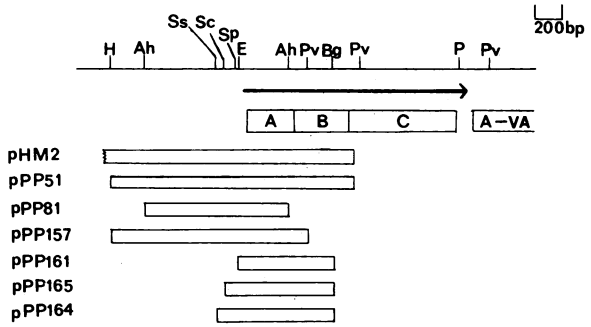

FIG. 1. Restriction endonuclease map of the *spoIIA* region of the *B. subtilis* chromosome (11), showing the portions present in plasmids used in the study; the full extent of the insert in pHM2 is not shown. The size and orientation of the *spoIIA* transcript are indicated by an arrow. A, B, and C represent the *spoIIA* open reading frames (11) coded for by genes *spoIIAA*, *spoIIAB*, and *spoIIAC*, respectively, within the *spoIIA* locus. A-VA represents the first open reading frame of the *spoVA* locus (10). Restriction endonuclease sites: Ah, *Aha*III; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; Sc, *Sca*I; Sp, *Spe*I; Ss, *Ssp*I. bp, Base pairs.

Primer extension. Primer extension analysis was by the method of Inoue and Cech (16). The synthetic primer (16-mer or 18-mer) was labeled at the 5′ ends by incubation at 37°C for 60 min with T4 polynucleotide kinase (New England Biolabs) in the presence of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham Corp.). A 50-μg sample of RNA was mixed with 100 ng of 5′ end-labeled primer and 10 U of RNasin (Promega Biotec) in a final volume of 20 μl of hybridization buffer (0.1 M KCl, 0.05 M Tris hydrochloride [pH 8.3]) and incubated at 30°C for 16 h. Potassium acetate was added to 0.3 M, and then nucleic acids were precipitated by the addition of 2 volumes of ethanol. The precipitate was collected by centrifugation, dried, and then suspended in 5 μl of diethylpyrocarbonate-treated water. To this was added 3.5 U of avian myeloblastosis virus reverse transcriptase (Promega Biotec), 10 U of RNasin, and 2 μl of 5× reverse transcriptase buffer (250 mM Tris hydrochloride, 200 mM KCl, 36 mM magnesium acetate, 10 mM dithiothreitol, 1 mM deoxynucleoside triphosphate, 4 U of RNasin). The reaction mixture was incubated at 42°C for 1 h. Primer-extended products were precipitated with 2 volumes of ethanol, washed with 70% (vol/vol) ethanol, dried, and dissolved in 4 μl of H_2O. Then 2 μl of gel loading buffer (80% [wt/vol] formamide, 1 mM EDTA [pH 8.0], 0.1% bromophenol blue, 0.1% xylene cyanol) was added. Samples were heated for 3 min at 90°C, and 3 μl was loaded onto an 8 M urea–6.0% (Fig. 2) or –7.5% (Fig. 3) polyacrylamide sequencing gel. The same primer was used for dideoxy DNA sequencing reactions with pPP157 as the template, and the products were loaded onto the gels to identify the initiating nucleotide.

Copy number determination. The number of copies of plasmid reiterated in the chromosome was determined as described previously (28) by using pulsed-field gel electrophoresis (1, 3). High-molecular-weight DNA extracted from the clones being tested was digested with a restriction endonuclease (*Bgl*II) that cut outside the reiterated region; from the size of the resulting *Bgl*II fragment, detected by Southern hybridization (31) with pPP81 as a probe (labeled by the method of Feinberg and Vogelstein [7]), the number of copies of reiterated plasmid was determined (28). The DNA preparations were also analyzed with an enzyme (*Eco*RI) that cut within the reiterated region to test for any deviation from the presumed structure of the integrated plasmid; that is, integration by a Campbell-like mechanism followed by the formation of precise tandem repeats of the integrated plasmid. In none of the clones discussed in this paper was any such deviation detected.

β-Galactosidase activity. Bacteria were permeabilized with toluene and assayed with *o*-nitrophenyl-β-D-galactopyranoside as substrate by the method of Miller (22). Specific activity is expressed as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of bacterial dry weight.

Sporulation. Individual clones were induced to sporulate in modified Schaeffer sporulation medium as described previously (29). In experiments involving Spo$^-$ mutants, con-

trol cultures of Spo$^+$ strains were included to ensure that the particular batch of medium used permitted good (greater than 60% of all organisms were phase-bright spores) sporulation. Samples were taken approximately 1 h before the end of exponential growth to be used for DNA extractions to measure plasmid copy number. Growth was followed by measuring the A_{600} and converting this to milligram bacterial dry weight per milliliter with a standard calibration curve determined for the spectrophotometer used.

Transformation. *E. coli* was transformed by a method of Hanahan (14). *B. subtilis* was transformed by the method of Piggot et al. (29); selection for chloramphenicol resistance was at 5 μg of chloramphenicol per ml.

Restriction endonucleases. Restriction endonucleases were obtained from Promega Biotec or from New England Biolabs. For conventional DNA preparations they were used according to the instructions of the manufacturer. For preparations of DNA embedded in agarose beads, they were used as described previously (28).

All other methods have been described previously (28).

RESULTS

Transcription start site. The primer extension method was used to determine the transcription start site. RNA was extracted at the end of exponential growth (defined as the start of sporulation, t_0) and at hourly intervals thereafter (t_1, t_2, etc.). Reverse transcripts were obtained with an 18-mer primer designed to hybridize to part of the mRNA coding for the *spoIIAA* gene, which is the promoter-proximal gene in the *spoIIA* locus (Fig. 1). The reverse transcripts were characterized by electrophoresis with respect to the products of dideoxy sequencing reactions obtained with the same primer and pPP157, which includes all of *spoIIAA* and more than 1 kb of its 5′-flanking sequence (Fig. 2). A strong *spoIIA* transcript signal was obtained for samples from t_2 to t_5. Two bands 3 bases apart were seen, and from these the transcription start was taken to be indicated by the stronger upper band. The same transcription start point was obtained with a second primer, a 16-mer from a region approximately 110 bases upstream from the first primer (Fig. 3). The 16-mer primer gave an additional band in early samples, suggesting a second transcription start point 16 bases downstream from the first start point. As this additional band was not seen with the same RNA preparations with the 18-mer primer, its significance is questionable. The transcription obtained with both primers corresponds to an A in the nontranscribed DNA strand (Fig. 4).

Deletion mapping of the *spoIIA* promoter. Integrational plasmids were used to delimit the size of the *spoIIA* control region (29). The plasmids were constructed by cloning into the vector pJM783 inserts of *B. subtilis* DNA that included part of the *spoIIA* coding region and extended upstream, to different extents, of the *spoIIA* transcription start point (Fig. 1). The plasmids replicated autonomously in *E. coli* but not in *B. subtilis*. When transformed into *B. subtilis* with selection for the vector-determined chloramphenicol resistance, transformants arose because of integration of the plasmid into the homologous region of the recipient chromosome by a Campbell-like mechanism. When the region cloned on an integrational plasmid was entirely internal to the *spoIIA* transcriptional unit (including its upstream *cis*-acting regulatory regions), then it disrupted that transcriptional unit (Fig. 5). When one end of the cloned region extended beyond the *spoIIA* transcriptional unit, then integration by a Campbell-like mechanism recreated an intact copy of the *spoIIA*

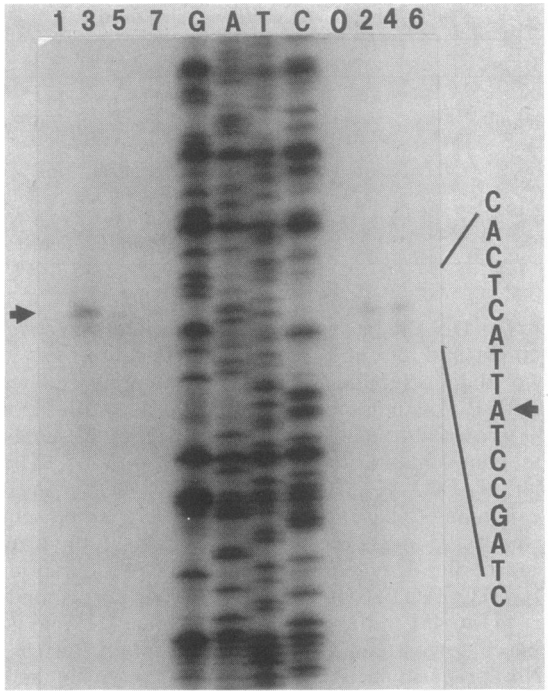

FIG. 2. Determination of the start point of *spoIIA* transcription with an 18-mer primer. Primer extension analysis was carried out on mRNA preparations as described in Materials and Methods. RNA was extracted from MB24 at the start of sporulation (sample 0) and at hourly intervals thereafter (samples 1 through 7). A sequencing ladder using the same primer is also shown. The letters above the lanes indicate which dideoxynucleotide was used to terminate the sequencing reaction. The sequence indicated is of the nontranscribed strand and is the complement of the sequence that can be read from the sequencing ladder. The transcription start site is indicated with an arrow.

transcriptional unit (Fig. 5). In this way the region upstream to at least the *Eco*RI site at −31 has previously been shown to be essential (29).

Plasmids were constructed which included *spoIIAA* DNA and extended upstream of the transcription start point to −52 (pPP161), −168 (pPP165), and −233 (pPP164) (Fig. 1). They were transformed into the Spo$^+$ strain MB24, selecting for chloramphenicol resistance. Plasmid pPP164 and pPP165 gave Spo$^+$ colonies, whereas plasmids pPP161 gave Spo$^-$ colonies. Transformant colonies were initially scored on the basis of colony pigmentation, and this scoring was confirmed by examination with a phase-contrast microscope and by testing for formation of heat-resistant spores in liquid culture. MB24::pPP164 and MB24::pPP165 gave 74 and 85% spore formation, respectively, whereas MB24::pPP161 gave less than 0.01% spore formation in liquid culture. Analysis by Southern hybridization of *Eco*RI-digested DNA extracted from 20 transformants from the pPP161 and pPP165 crosses confirmed that integration was by a Campbell-like mechanism. Deleting material upstream of −52 also abolished promoter activity as judged by the lack of β-galactosidase activity of a *spoIIA-lacZ* transcriptional fusion (data not shown). We concluded that the *spoIIA* control region extends at least to −52, and that the region upstream of −168 is not necessary to get sufficient *spoIIA* expression to obtain maximal spore formation (Fig. 4); it remains formally possible that regions further upstream are needed to obtain maximal *spoIIA* expression.

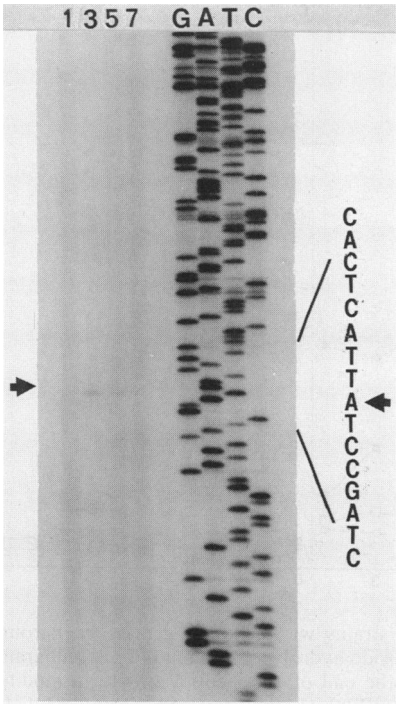

FIG. 3. Determination of the start point of *spoIIA* transcription with a 16-mer primer. Primer extension analysis was carried out as described in Materials and Methods. RNA was extracted from MB24 at 1, 3, 5, and 7 h after the start of sporulation. A sequencing ladder using the same primer is also shown. The sequence indicated is of the complement of the sequence read from the sequencing ladder.

Effect of *spo0* mutations on *spoIIA* expression. A *spoIIA-lacZ* transcriptional fusion in an integrational plasmid, pPP81, was used to study the effects of *spo0* mutations on *spoIIA* expression. Strains were used in which the plasmid had integrated by a Campbell-like mechanism into the chromosome. The pPP81 construct includes the 5′ end of the *spoIIA* transcriptional unit and so does not disrupt sporulation of *spo*$^+$ strains (26). In confirmation of previous results (26, 28), a Spo$^+$ strain (MB24) containing a single copy of pPP81 synthesized β-galactosidase beginning about 60 to 90 min after the start of sporulation (Fig. 6A). This induction was prevented by mutations in the *spo0A* locus (strains SL566::pPP81 and JH646::pPP81; Fig. 6A). Mutations in the *abrB* locus suppress some of the defects resulting from *spo0A* mutations (24, 36), but no suppression was seen with the *abr4* mutation (strain SL703abr4::pPP81; Fig. 6A). The *spo0A* block was partly overcome by having multiple copies of the *spoIIA-lacZ* fusion. Even with 14 copies, however, enzyme activity was less than that found for a Spo$^+$ strain containing a single copy of the fusion (Fig. 6A). Clones with multiple copies were obtained by selecting (at 40 to 80 μg of chloramphenicol per ml) for tandem repeats of the integrated plasmid (30). Ten other *spo0A34* clones, two *spo0A12* clones, and two *spo0A677 abr4* clones selected in this way were tested; none gave greater β-galactosidase activity than did a *spo*$^+$ strain with a single *spoIIA-lacZ* fusion (data not shown).

Mutations in the *spo0B* (strain SL964) and *spo0F* (strain SL965) loci also prevented the large induction of β-galactosidase obtained with the *spo*$^+$ strain (MB24) when a single *spoIIA-lacZ* copy was present (Fig. 6B). The *spo0B* block appeared less complete than the *spo0F* block and was overcome to a greater extent than the *spo0F* (or *spo0A*) block when multiple copies of the *spoIIA-lacZ* fusion were present (Fig. 6B); even with *spo0B*, expression did not exceed that obtained with a single copy of the fusion in a Spo$^+$ strain (Fig. 6A and B). In contrast, the *spo0H17* mutation (strain SL513) prevented β-galactosidase expression even when 30 copies of the fusion were present (Fig. 6C). Six other clones with multiple copies of pPP81 in SL513

```
-240       -230       -220       -210       -200       -190
 *          *          *          *          *          *
GAAGGACAATATTAGTGCTCCGATTCAAAAAGGCCAAGAGCTTGGCACTCTTGTTCTGAA
          ^SspI

-180       -170       -160       -150       -140       -130
 *          *          *          *          *          *
AAAGGATGGAGAAGTACTCGCTGAAAGTCCTGTTGCTGCAAAAGAAGATATGAAGAAAGC
                ^ScaI

-120       -110       -100        -90        -80        -70
 *          *          *          *          *          *
CGGGTTTATCACATTCTTAAAGCGGACGATGGGAGACTGGACAAAATTTAAGTAATTATG

 -60        -50        -40        -30        -20        -10
 *          *          *          *          *          *
CCGAATGACCACTAGTTTTGTCACGGTGAAGGAATTCATTCCGTCGAAATCGAAACACTC
          ^SpeI                   ^EcoRI

 +1
 *
ATTATCCGATCATATCAAGGAGGAATGAGCATGAGCCTTGGAATTGACATGAATGTCAAA
              RBS          MetSerLeuGly.....
```

FIG. 4. DNA sequence of the nontranscribed strand of the *spoIIA* promoter region. The transcription start point is shown as +1. The putative ribosome-binding site for the *spoIIAA* gene is underlined, and the putative N-terminal sequence of the SpoIIAA protein is given (11). Restriction sites in the promoter region are also shown. The arrowheads indicate the positions in the region that were tested when defining the transcriptional unit with integrational plasmid.

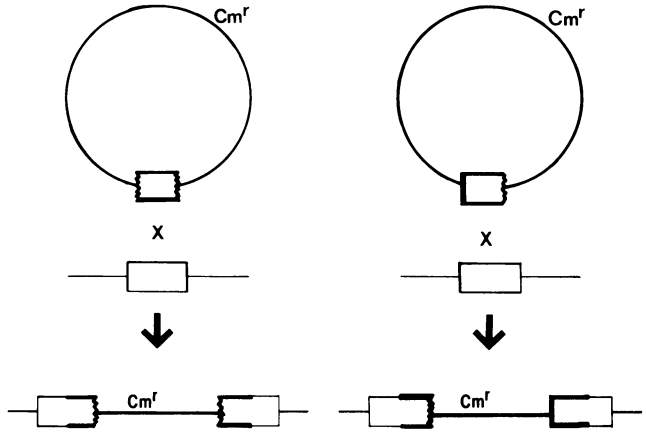

FIG. 5. Illustration of the integration of a plasmid into the chromosome by a Campbell-type recombination in the region of homology between the chromosome and DNA cloned on the plasmid. The plasmid is drawn in bold lines; the chromosome is drawn in thin lines. A complete transcriptional unit, including all essential *cis*-acting regulatory regions, is shown as a rectangle with straight ends. Incomplete transcriptional units are indicated by serrated ends on a rectangle. On the left the region cloned on the plasmid is entirely internal to the transcriptional unit, and integration yields no complete transcriptional unit. On the right the cloned region includes one end of the transcriptional unit, and integration recreates one intact transcriptional unit; when the 5′ end of the unit is intact in the clone, the downstream transcription unit is intact upon integration. The location of the plasmid determined chloramphenicol resistance is indicated by Cm^r.

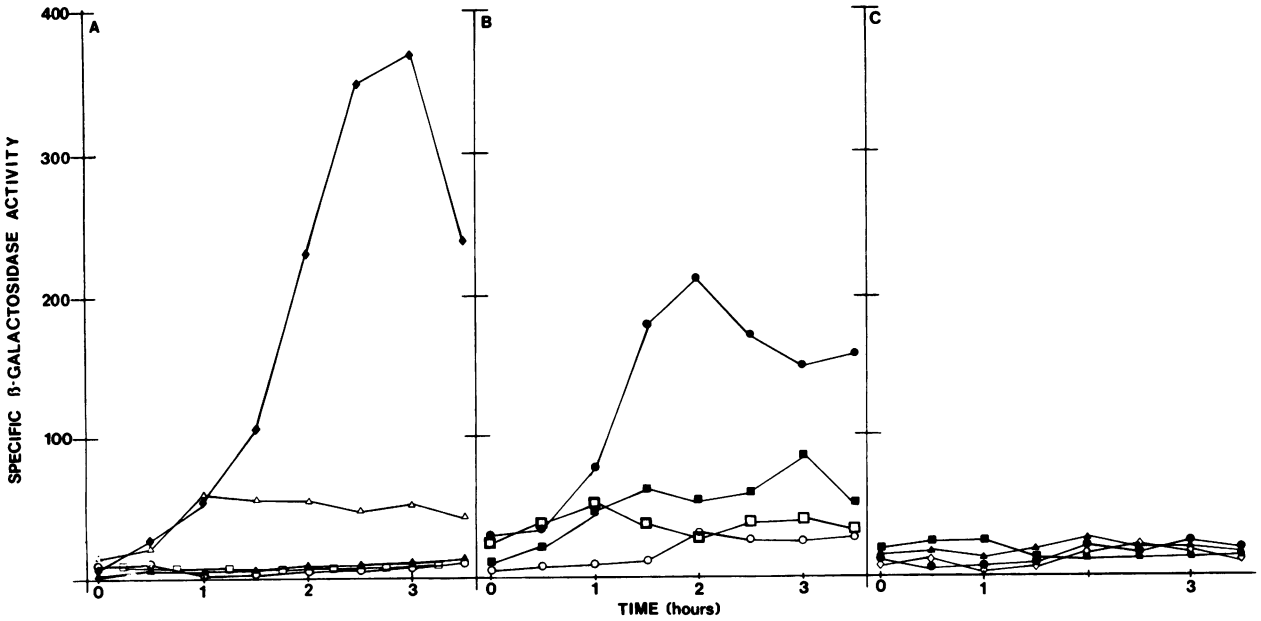

FIG. 6. Formation of β-galactosidase by a *spoIIA-lacZ* transcriptional fusion in strains with different genetic backgrounds. Specific β-galactosidase activity is expressed as nanomoles of σ-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of bacterial dry weight. The time is the time after the start of sporulation, which is defined as the end of exponential growth in modified Schaeffer sporulation medium. (A) Symbols: ◆, MB24::pPP81 (1 plasmid copy); ▲, SL566::pPP81 (1 copy); △, SL566::pPP81 (14 copies); □, JH646::pPP81 (1 copy); ○, JH703*abr4*::pPP81 (1 copy). (B) Symbols: ○, SL964::pPP81 (1 copy); ●, SL964::pPP81 (25 copies); □, SL965::pPP81 (1 copy); ■, SL965::pPP81 (18 copies). (C) Symbols: ▲, SL513::pPP81 (1 copy); ●, SL513::pPP81 (17 copies); ■, SL513::pPP81 (30 copies); ◇, MB24 (no plasmid).

gave the same result. The β-galactosidase activity of these strains was comparable to the very low endogenous activity of strain MB24 containing no *spoIIA-lacZ* fusion (Fig. 6C). It was necessary to rule out the possibility that mutations within the *spoIIA-lacZ* region had caused the lack of β-galactosidase activity in the SL513::pPP81 clones. To do this, DNA preparations from the SL513::pPP81 clones were used to transform the *spo*$^+$ strain MB24 to chloramphenicol resistance. Transformants were then tested for β-galactosidase production and in all cases produced it (data not shown). We concluded that the *spoIIA-lacZ* fusion was intact in the SL513::pPP81 clones and that their lack of β-galactosidase activity was a consequence of the *spo0H17* mutation.

DISCUSSION

The start site of *spoIIA* transcription has been defined by primer extension with two different primers that were complementary to regions of the *spoIIA* mRNA separated by about 110 bases. The transcription start point was 27 bases upstream from the likely translation initiation codon (Fig. 4).

Transcription of *spoIIA* is dependent on the *spo0A*, *spo0B*, *spo0F*, and *spo0H* loci (6, 26, 33, 34). The dependence on *spo0A*, *spo0B*, and *spo0F* could be partly overcome by having multiple copies of the *spoIIA* promoter region, but dependence on *spo0H* could not (Fig. 6). This dependence pattern is reminiscent of that of the *spoVG* locus (38, 39). The *spoVG* locus is transcribed by RNA polymerase containing sigma factor H, σ^H (2, 38), which is coded for by the *spo0H* locus (4). (This RNA polymerase holoenzyme is abbreviated to E-σ^H.) The region upstream of the *spoIIA* transcription start point was found to have extensive homology to the −35 and −10 regions of E-σ^H promoters (Fig. 7). Thus, there is reasonable circumstantial evidence that *spoIIA* is transcribed by E-σ^H. Without evidence such as in vitro transcription and allele-specific suppression (15), such a conclusion is, of course, tentative. Control of *spoIIA* transcription differs from that of *spoVG* because the effect of a *spo0A* mutation is not overcome by an *abrB* mutation (39). It also appears to differ from the control of *spoIIE* and *spoIIG* transcription, which is thought to be mediated by E-σ^A (13, 19).

If *spoIIA* is, in fact, transcribed by E-σ^H, then E-σ^H is unlikely to be the only control of *spoIIA* expression. σ^H is present during vegetative growth (2, 4), whereas *spoIIA* transcription starts about 1 h after the start of sporulation. On increasing the number of copies of a *spoIIA-lacZ* fusion in a Spo$^+$ strain, formation of β-galactosidase is maximal at about 4 copies, and yet spore formation is not greatly impaired even when there are more than 20 copies (28); this would not be expected (although it is not impossible) if E-σ^H were the only, and hence the limiting, factor in *spoIIA* expression, since E-σ^H is also required for the transcription of other genes, most notably *spoVG*. The roles of *spo0A*,

```
                -30        -20        -10        +1
                                                 *
spoIIA     GTGAAGGAATTCATTCCGTCGAAATCGAAACACTCATTATC
                                                 *
rpoD P3    ATGCAGGAGTTTAATGGAGGGATGGAGAATTACTCTTCTTA
                                                 *
spoVG P1   GAGCAGGATTTCAGAAAAAAT CGTGGAATTGATACACTAA
```

FIG. 7. Nucleotide sequence of the *spoIIA* promoter region compared to the sequences of *spoVG* P1 and *rpoD* P3, promoters that are utilized by E-σ^H (2). The start points of transcription are indicated with asterisks (*). Numbering is for *spoIIA*, with +1 the start of transcription. Homologous regions at −10 and −35 are underlined.

FIG. 8. Nucleotide sequence of the nontranscribed strand of the putative *spoIIA* transcription terminator. The last codon of the *spoIIAC* gene (5) is underlined. The number of nucleotides from the transcription start point is indicated.

spo0B, and *spo0F* in *spoIIA* transcription are unclear, but appear to be indirect. It appeared that the *spo0B* mutation could be overcome to a greater extent than *spo0A* or *spo0F* mutations by having multiple *spoIIA-lacZ* copies, but in no case was the effect of the *spo0* mutation totally overcome. The restored *spoIIA-lacZ* expression in *spo0* mutants showed normal temporal control.

The first open reading frame of the *spoVA* locus is located about 220 bases downstream from the last open reading frame of the *spoIIA* locus (10), and *spoVA* is transcribed separately from the *spoIIA* locus (29). A possible terminator for *spoIIA* transcription is located immediately after the end of the last *spoIIA* open reading frame (Fig. 8). The distance from the transcription start site to this putative transcription terminator is 1,619 bases. This agrees well with the estimate from Northern blots by Savva and Mandelstam (33, 34) of 1.4 to 1.7 kb for the size of *spoIIA* mRNA; our own estimate from Northern blots was 1.7 kb (unpublished observations). The time of appearance of this transcript, 1 to 2 h after the start of the sporulation (33, 34), agrees with the time of appearance of the transcript identified by primer extension (Fig. 2) and the time of expression of *spoIIA-lacZ* transcriptional fusions (6, 26, 28) (Fig. 6). Savva and Mandelstam also noted a larger, 2.6-kb, *spoIIA*-hybridizing transcript that appears about 3 h after the start of sporulation and whose transcription starts at least 700 bases upstream from the start of the 1.7-kb transcript; we have also detected this transcript in Northern blots (unpublished observations). The studies with integrational plasmids reported here establish that the larger transcript cannot be necessary for sporulation, and it was not investigated further.

ACKNOWLEDGMENTS

We thank Kim de Riel for the construction of the oligonucleotide primers; Brian Moldover, Rich Losick, Charlie Moran, and Kim de Riel for helpful discussions; and Greg Harvey for help in preparation of the manuscript.

This work was supported by Public Health Service grant AI23045 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Carle, G. F., and M. V. Olson.** 1984. Separation of chromosomal DNA molecules from yeast by orthogonal-field-alternation gel electrophoresis. Nucleic Acids Res. **12**:5647–5664.
2. **Carter, H. L., III, and C. P. Moran, Jr.** 1986. New RNA polymerase sigma factor under *spo0* control in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **83**:9438–9442.
3. **Chu, G., D. Vollrath, and R. W. Davis.** 1986. Separation of large DNA molecules by contour clamped homogeneous electrical fields. Science **234**:1582–1585.
4. **Dubnau, E., H. Cabane, and I. Smith.** 1987. Regulation of *spo0H*, an early sporulation gene in bacilli. J. Bacteriol. **169**: 1182–1191.
5. **Errington, J., P. Fort, and J. Mandelstam.** 1985. Duplicated sporulation genes in bacteria. Implications for simple developmental systems. FEBS Lett. **188**:184–188.
6. **Errington, J., and J. Mandelstam.** 1986. Use of a *lacZ* gene fusion to determine the dependence pattern of sporulation operon *spoIIA* in *spo* mutants of *Bacillus subtilis*. J. Gen. Microbiol. **132**:2967–2976.
7. **Feinberg, A. P., and B. Vogelstein.** 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Addendum. Anal. Biochem. **137**:266–267.
8. **Ferrari, E., D. J. Henner, M. Perego, and J. A. Hoch.** 1988. Transcription of *Bacillus subtilis* subtilisin and expression of subtilisin in sporulation mutants. J. Bacteriol. **170**:289–295.
9. **Ferrari, F. A., A. Nguyen, D. Lang, and J. A. Hoch.** 1983. Construction and properties of an integrable plasmid for *Bacillus subtilis*. J. Bacteriol. **154**:1513–1515.
10. **Fort, P., and J. Errington.** 1985. Nucleotide sequence and complementation analysis of a polycistronic sporulation operon, *spoVA*, in *Bacillus subtilis*. J. Gen. Microbiol. **131**:1091–1105.
11. **Fort, P., and P. J. Piggot.** 1984. Nucleotide sequence of sporulation locus, *spoIIA*, in *Bacillus subtilis*. J. Gen. Microbiol. **130**:2147–2153.
12. **Guerry, P., D. J. LeBlanc, and S. Falkow.** 1973. General method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. **116**:1064–1066.
13. **Guzman, P., J. Westpheling, and P. Youngman.** 1988. Characterization of the promoter region of the *Bacillus subtilis spoIIE* operon. J. Bacteriol. **170**:1598–1609.
14. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
15. **Helmann, J. D., and M. J. Chamberlin.** 1988. Structure and function of bacterial sigma factors. Annu. Rev. Biochem. **57**: 839–872.
16. **Inoue, T., and T. R. Cech.** 1985. Secondary structure of the circular form of the *Tetrahymena* rRNA intervening sequence: a technique for RNA structure analysis using chemical probes and reverse transcriptase. Proc. Natl. Acad. Sci. USA **82**:648–652.
17. **Ionesco, H., J. Michel, B. Cami, and P. Schaeffer.** 1970. Genetics of sporulation in *Bacillus subtilis* Marburg. J. Appl. Bacteriol. **33**:13–24.
18. **Ish-Horowicz, D., and J. F. Burke.** 1981. Rapid and efficient cosmid cloning. Nucleic Acids Res. **9**:2989–2998.
19. **Kenney, T. J., P. A. Kirchman, and C. P. Moran, Jr.** 1988. Gene encoding σ^E is transcribed from a σ^A-like promoter in *Bacillus subtilis*. J. Bacteriol. **170**:3058–3064.
20. **Liu, H. M., K. F. Chak, and P. J. Piggot.** 1982. Isolation and characterization of a recombinant plasmid carrying a functional part of the *Bacillus subtilis spoIIA* locus. J. Gen. Microbiol. **128**:2805–2812.
21. **Losick, R., P. Youngman, and P. J. Piggot.** 1986. Genetics of endospore formation in *Bacillus subtilis*. Annu. Rev. Genet. **20**:625–669.
22. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Penn, M. D., G. Thireos, and H. Greer.** 1984. Temporal analysis of general control of amino acid biosynthesis in *Saccharomyces cerevisiae*: role of positive regulatory genes in initiation and maintenance of mRNA depression. Mol. Cell. Biol. **4**:520–528.
24. **Perego, M., and J. Hoch.** 1988. Molecular cloning of the transcription inhibitor *abrB* of *Bacillus subtilis*, p. 129–134. *In* A. T. Ganesan and J. A. Hoch (ed.), Genetics and biotechnology of bacilli, vol. 2. Academic Press, Inc., San Diego, Calif.
25. **Piggot, P. J.** 1973. Mapping of asporogenous mutations of *Bacillus subtilis*: a minimum estimate of the number of sporulation operons. J. Bacteriol. **114**:1241–1253.
26. **Piggot, P. J., J. W. Chapman, and C. A. M. Curtis.** 1985. Analysis of the control of *spo* gene expression in *Bacillus subtilis*, p. 15–21. *In* J. A. Hoch and P. Setlow (ed.), Molecular biology of microbial differentiation. American Society for Microbiology, Washington, D.C.
27. **Piggot, P. J., and J. G. Coote.** 1976. Genetic aspects of bacterial endospore formation. Bacteriol. Rev. **40**:908–962.
28. **Piggot, P. J., and C. A. M. Curtis.** 1987. Analysis of the regulation of gene expression during *Bacillus subtilis* sporulation by manipulation of the copy number of *spo-lacZ* fusions. J. Bacteriol. **169**:1260–1266.
29. **Piggot, P. J., C. A. M. Curtis, and H. de Lencastre.** 1984. Use of integrational plasmid vectors to demonstrate the polycistronic

nature of a transcriptional unit (*spoIIA*) required for sporulation of *Bacillus subtilis*. J. Gen. Microbiol. **130**:2123–2136.

30. **Piggot, P. J., J.-J. Wu, C. A. M. Curtis, and J. W. Chapman.** 1988. Manipulation of gene copy number in *Bacillus subtilis* using integrative plasmids, p. 141–145. *In* A. T. Ganesan and J. A. Hoch (ed.), Genetics and biotechnology of bacilli, vol. 2. Academic Press, Inc., San Diego, Calif.
31. **Reed, K. C., and D. A. Mann.** 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. **13**: 7207–7221.
32. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
33. **Savva, D., and J. Mandelstam.** 1985. Use of cloned *spoIIA* and *spoVA* probes to study synthesis of mRNA in wild-type and asporogenous mutants of *Bacillus subtilis*, p. 55–59. *In* J. A. Hoch and P. Setlow (ed.), Molecular biology of microbial differentiation. American Society of Microbiology, Washington, D.C.
34. **Savva, D., and J. Mandelstam.** 1986. Synthesis of *spoIIA* and *spoVA* mRNA in *Bacillus subtilis*. J. Gen. Microbiol. **132**: 3005–3011.
35. **Trempy, J. E., C. Bonamy, J. Szulmajster, and W. G. Haldenwang.** 1985. *Bacillus subtilis* sigma factor σ^{29} is the product of the sporulation-essential gene *spoIIG*. Proc. Natl. Acad. Sci. USA **82**:4189–4192.
36. **Trowsdale, J., S. M. H. Chen, and J. A. Hoch.** 1979. Genetic analysis of a class of polymyxin resistant partial revertants of state 0 sporulation mutants of *Bacillus subtilis*: map of the chromosome region near the origin of replication. Mol. Gen. Genet. **173**:61–70.
37. **Zuber, P., J. M. Healy, and R. Losick.** 1987. Effect of plasmid propagation of a sporulation promoter on promoter utilization and sporulation in *Bacillus subtilis*. J. Bacteriol. **169**:461–469.
38. **Zuber, P., and R. Losick.** 1983. Use of a lacZ fusion to study the role of the *spo0* genes of *Bacillus subtilis* in developmental regulation. Cell **35**:275–283.
39. **Zuber, P., and R. Losick.** 1987. Role of AbrB in Spo0A- and Spo0B-dependent utilization of a sporulation promoter in *Bacillus subtilis*. J. Bacteriol. **169**:2223–2230.