JOURNAL OF BACTERIOLOGY, May 1991, p. 2977–2984
0021-9193/91/092977-08$02.00/0

Vol. 173, No. 9

Control of Transcription of the *Bacillus subtilis spoIIIG* Gene, Which Codes for the Forespore-Specific Transcription Factor σ^G

DONGXU SUN, ROSA MARTHA CABRERA-MARTINEZ, AND PETER SETLOW*

Department of Biochemistry, University of Connecticut Health Center, Farmington, Connecticut 06032

Received 25 September 1990/Accepted 11 November 1990

The *Bacillus subtilis spoIIIG* gene codes for a sigma factor termed σ^G which directs transcription of genes expressed only in the forespore compartment of the sporulating cell. Use of *spoIIIG-lacZ* transcriptional fusions showed that *spoIIIG* is cotranscribed with the *spoIIG* operon beginning at $t_{0.5-1}$ of sporulation. However, this large mRNA produced little if any σ^G, and transferring the *spoIIIG* gene without the *spoIIG* promoter into the *amyE* locus resulted in a Spo$^+$ phenotype. Significant translation of *spoIIIG* began at $t_{2.5-3}$ with use of an mRNA whose 5′ end is just upstream of the *spoIIIG* coding sequence. Synthesis of this *spoIIIG*-specific mRNA was not abolished by a deletion in *spoIIIG* itself. Similar results were obtained when a *spoIIIG-lacZ* translational fusion lacking the *spoIIG* promoter was integrated at the *amyE* locus. These data suggest that synthesis of σ^G is dependent neither on transcription from the *spoIIG* promoter nor on σ^G itself but can be due to another transcription factor. This transcription factor may be σ^F, the product of the *spoIIAC* locus, since a *spoIIAC* mutation blocked *spoIIIG* expression, and sequences upstream of the 5′ end of the *spoIIIG*-specific mRNA agree well with the recognition sequence for σ^F. RNA polymerase containing σ^F ($E\sigma^F$) initiated transcription in vitro on a *spoIIIG* template at the 5′ end found in vivo, as did $E\sigma^G$. However, $E\sigma^F$ showed a >20-fold preference for *spoIIIG* over a known σ^G-dependent gene compared with the activity of $E\sigma^G$.

Between h 1 and 2 of sporulation (t_{1-2}) in *Bacillus subtilis*, the sporulating cell undergoes an asymmetric division, with the resultant larger cell termed the mother cell and the smaller cell termed the forespore. As development proceeds, the mother cell engulfs the forespore, and eventually the mother cell lyses, releasing the free dormant spore. Work in a number of laboratories has shown that many genes are expressed in only one of the compartments of the sporulating cell (10, 11, 23, 25). When regulation of expression of such genes has been studied in detail, it has been found to be exerted at the transcriptional level, through the aegis of compartment-specific sigma factors for RNA polymerase (7, 9, 17).

The forespore-specific sigma factor is termed σ^G and is the product of the *spoIIIG* gene (7, 15, 26). This gene is located just downstream of the *spoIIG* operon (Fig. 1a) and appears to be transcribed from the *spoIIG* promoter beginning at about $t_{0.5-1}$ of sporulation (15). However, detectable synthesis of σ^G does not begin until t_{2-3}, probably by translation of an RNA synthesized at this time whose 5′ end is just prior to the *spoIIIG* translational start site (7, 15, 26). Strikingly, synthesis of σ^G appears confined to the forespore (7). Since RNA polymerase containing σ^G ($E\sigma^G$) directs expression of most forespore-specific genes, understanding the mechanism whereby σ^G synthesis is restricted to the forespore could explain the basis for compartment-specific gene expression. Clearly, it would be of interest to learn how the expression of *spoIIIG* is regulated, in particular the identity of the transcription factor directing synthesis of the short *spoIIIG*-specific mRNA which appears to direct most if not all σ^G synthesis. It has been suggested, on the basis of analysis of the expression of a *spoIIIG* transcriptional fusion as well as other data (7, 25), that *spoIIIG* expression is autoregulatory, i.e., that $E\sigma^G$ transcribes *spoIIIG*-specific mRNA, with the small amount of σ^G needed to begin this process coming from the mRNA initiated from the *spoIIG* promoter. While this regulatory scheme is possible, other mechanisms are feasible. Consequently, we have examined the regulation of *spoIIIG* in detail, in particular to determine the necessity of the *spoIIG* promoter for σ^G synthesis and the identity of the sigma factor directing synthesis of *spoIIIG*-specific mRNA.

MATERIALS AND METHODS

Bacteria, plasmids, and DNAs. The bacterial strains and plasmids used are listed in Table 1. *B. subtilis* and *Escherichia coli* strains were routinely grown at 37°C in 2×YT medium (16). Concentrations of antibiotics when used were as follows: ampicillin, 50 μg/ml; chloramphenicol, 3 μg/ml; kanamycin, 10 μg/ml; macrolides-lincosamides-streptrogramin S (MLS) erythromycin and lincomycin, 1 and 25 μg/ml, respectively. *B. subtilis* strains were sporulated at 37°C in 2×SG medium (6), and the beginning of sporulation (t_0) was defined as previously described (29). Plasmid and chromosomal DNA was extracted from *E. coli* and *B. subtilis* strains and purified if necessary as previously described (2). *E. coli* strains were made competent and transformed as described previously (2).

Analytical methods. *B. subtilis* RNA was extracted, purified, and analyzed by primer extension using DNA primers which were isolated by agarose gel electrophoresis of appropriate restriction enzyme digests of plasmid DNA or DNA fragments (12, 19). Specific primers used were the 38-bp *Taq*I-*Sca*I fragment and the 75-bp *Sca*I-*Hin*dIII fragment (Fig. 1a) (7, 15). The latter fragment was isolated initially as the 460-bp *Hin*dIII fragment, end labelled, and then cut with *Sca*I, and the 75-bp fragment was reisolated and used as a primer. Note that the *Sca*I-*Hin*dIII fragment will give no priming with RNA from a gene with the *spoIIIGΔ1* mutation, because the *Sca*I-*Hin*dIII region has been deleted (7). DNA sequence analysis using these primers was carried out by the chain termination method.

Samples of sporulating cells were harvested, treated with

* Corresponding author.

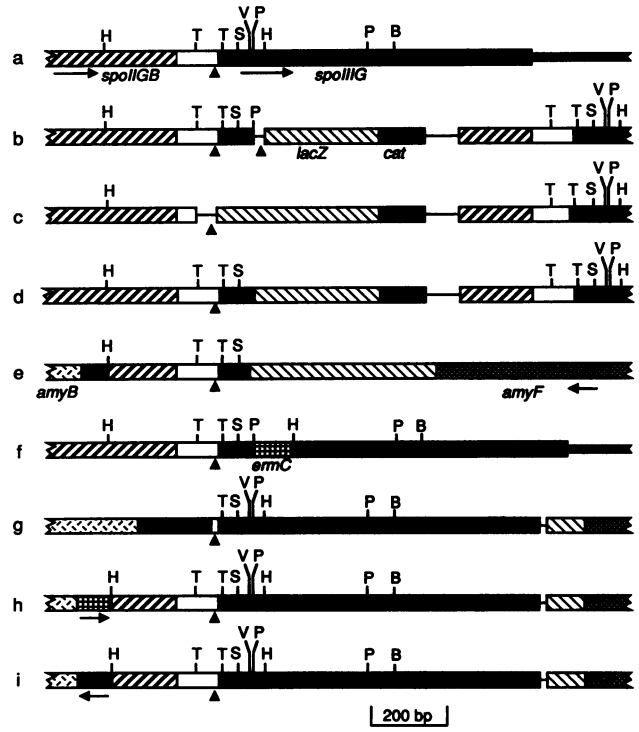

FIG. 1. Chromosomal structure of the *B. subtilis spoIIG-spoIIIG* or *amyE* locus with (b to i) or without (a) various plasmids integrated. Plasmids used for integration are described in Materials and Methods. The plasmids integrated at the *spoIIG-spoIIIG* locus are pPS956 (b), pPS955 (c), pPS896 (d), and pPS1110 (f). The plasmids integrated at the *amyE* locus are pPS939 (e), pPS996 (strain PS1095) (g), pPS1093 (integrated into strain PS1095, giving strain PS1096) (h), and pPS939 (integrated into strain PS1096, giving strain PS1120) (i). The various coding regions are labeled only the first time they are shown, but the same shading patterns are used throughout. The unshaded box is the *spoIIG-spoIIIG* intergenic region. *amyB* and *amyF* denote the back and front regions, respectively, of the *amyE* gene (24). The thin line denotes plasmid DNA other than the *cat*, *ermC*, or *lacZ* gene; the thick line denotes other *B. subtilis* chromosomal DNA. The scale for *spoIIG*, *spoIIIG*, and the *spoIIG-spoIIIG* intergenic region is constant throughout the figure and is that of the size marker shown. However, the sizes of the *cat*, *ermC*, and *lacZ* genes and plasmid DNA shown vary and are not to scale. The small amount of *lacZ* remaining in constructs g, h, and i is only a few hundred nucleotides from the C-terminal coding region. The arrows below coding regions denote the direction of transcription; the solid triangle denotes a ribosome binding site preceding *lacZ* or *spoIIIG*. Restriction sites: B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; S, *Sca*I; T, *Taq*I; V, *Pvu*II. Not all *Taq*I sites are shown.

lysozyme, and assayed for β-galactosidase and glucose dehydrogenase as previously described (13). Determination of heat-resistant spores was carried out as described by Mason and Setlow (14).

RNA polymerase containing $E\sigma^F$ but not $E\sigma^G$ was isolated at $\sim t_3$ from strain PS683, which carries a deletion in *spoIIIG*, and purified through the heparin agarose chromatography step (19, 26). RNA polymerase containing $E\sigma^G$ but not $E\sigma^F$ was isolated from strain PS749, which carries *spoIIIG* under *spac* promoter control and is thus inducible by isopropyl-β-D-thiogalactopyranoside (IPTG). $E\sigma^G$ was purified from vegetative cells of this strain, which were induced for 2 h with IPTG as previously described (19, 26). In vitro transcription reactions with these two forms of RNA polymerase were carried out with template in excess and adding equal amounts of enzyme [as measured by activity with poly(dA-dT)] as described by Nicholson et al. (19). Reactions were carried out without heparin and using [α-^{32}P]ATP and were initiated by addition of enzyme. Two templates were present in the reactions: (i) plasmid pPS591 (1 μg), which carries *sspE* (19) (when cut with *Eco*RI, this template gives a 194-nucleotide (nt) transcript with $E\sigma^G$ and $E\sigma^F$ [19]); and (ii) plasmid pTK4 (1.6 μg), which carries part of *spoIIG* and *spoIIIG* (8) cut with *Bam*HI. The transcripts were treated and analyzed by acrylamide gel electrophoresis as described previously (19, 26). The sizes of the transcripts were determined with reference to a series of RNA size markers (19) and were cut out and counted in a scintillation counter to quantitate transcription. Counts were corrected for background by counting a region of the gel from between the two transcripts (29).

TABLE 1. *B. subtilis* strains

Strain	Genotype	Source or reference
PS607	*trpC2 spoIIIE36*	I. Smith
M0428(PS683)	*trpC2 spoIIIGΔ1*	P. Stragier (7)
PS749	*pheA1 trpC2 spoA12 sspA-lacZ* Cm^r(pDG298 *spac-sigG* Km^r)	19
PS768	*trpC2 spoIIIGΔ1*(pDG298 *spac-sigG* Km^r)	pDG298→PS683
168(PS832)	*trpC2*	Laboratory stock
PS1096	*trpC2 spoIIIGΔ1 amyE*:: *spoIIIG* MLS^r	This work
PS1120	*trpC2 spoIIIGΔ1 amyE*:: *spoIIIG* Cm^r	This work
PS1128	*trpC2 spoIIIE36 spoIIIG*:: p1110 MLS^r	pPS1110→PS607
PS1175	*trpC2 spoIIAC1*	J. Errington

Construction of *spoIIIG* transcriptional fusions. To construct transcriptional *lacZ* fusions to *spoIIIG*, we first constructed a transcriptional fusion vector termed pJF751A. This was accomplished by cleaving the translational *lacZ* fusion vector pJF751 (4) with *Eco*RI and *Cla*I and replacing this 0.8-kb fragment with the analogous *Eco*RI-*Cla*I fragment from pDG268 (1). The resulting plasmid, pJF751A, carries a promoterless *lacZ* gene with a good ribosome binding site and can replicate in *E. coli* but not in *B. subtilis*. Consequently, it will confer stable drug resistance in *B. subtilis* only if it integrates into the chromosome. Plasmid pTK4 (8), which carries a part of the *spoIIGB* and *spoIIIG* genes (Fig. 1a), was cut with *Hin*dIII and *Pst*I, and the 0.43-kb fragment was isolated. Some of this fragment was cloned into *Hin*dIII-*Pst*I-cut pUC12, and the resulting 0.44-kb *Hin*dIII-*Bam*HI fragment from one clone was inserted into *Hin*dIII-*Bam*HI-cut pJF751A, generating pPS956 (Fig. 1b). This plasmid carries part of the *spoIIGB* and *spoIIIG* coding sequences prior to *lacZ*. The remaining 0.44-kb *Hin*dIII-*Pst*I fragment was cut with *Taq*I, and the 0.25-kb *Hin*dIII-*Taq*I fragment was cloned into *Hin*dIII-*Acc*I-cut pUC12. The 0.26-kb *Hin*dIII-*Bam*HI fragment from one transformant was cloned into *Hin*dIII-*Bam*HI-cut pJF751A, giving pPS955 (Fig. 1c). This plasmid has only a part of the *spoIIGB* coding sequence and the *spoIIG-spoIIIG* intergenic region preceding the *lacZ* gene. When these plasmids integrate into the *B. subtilis* chromosome (Fig. 1b and c), a complete *spoIIIG* gene remains but is moved ~7.5 kb further downstream of the *spoIIG* promoter.

Construction of translational *spoIIIG-lacZ* fusions. The 0.43-kb *Hin*dIII-*Pvu*II fragment from pTK4 was isolated, and the ends were filled with *E. coli* DNA polymerase and inserted into pJF751 cut with *Sma*I. Plasmid with the correct orientation was identified by digestion with *Cla*I and *Sca*I, giving rise to pPS896, which has the *spoIIIG* gene in frame with *lacZ* (Fig. 1d). Again, when this plasmid integrates into the chromosome, an intact *spoIIIG* remains but is moved downstream (Fig. 1d).

The 1.25-kb *Eco*RI-*Cla*I fragment from pPS896 was isolated and cloned in ptrpBGI (24) cut with *Eco*RI and *Cla*I, giving pPS939. This plasmid, like the pJF751 derivatives, cannot replicate in *B. subtilis*, but it can integrate not only at the locus of any *B. subtilis* DNA carried but also at the *amyE* locus (Fig. 1e). Such integration renders the cell amylase negative, a phenotype which is easily detected (24). Note that the *amyE* promoter is in the orientation opposite that of *spoIIIG* transcription (Fig. 1e) (24).

Construction of a *spoIIIG* integration inactivation vector. Plasmid pTK4 was cut with *Pvu*II and *Bam*HI, and the 0.41-kb *Pvu*II-*Bam*HI fragment from pDG298 (29) was inserted, forming pPS1008. This introduces an additional 0.4 kb of *spoIIIG* coding sequence into pTK4. Plasmid pPS1008 was cut with *Eco*RI and *Cla*I, and a 1.2-kb *Eco*RI-*Cla*I fragment containing the *ermC* gene was inserted, giving pPS1092. The *ermC* gene, which came from plasmid pE194, was excised as a 1.4-kb *Hpa*II fragment and cloned in the *Acc*I site of pUC12 with the direction of transcription of the *ermC* gene from the *Eco*RI site toward the *Hin*dIII site in the polylinker. Plasmid pPS1092 was cut with *Hin*dIII, the 0.5-kb *Hin*dIII fragment was removed, and the plasmid was religated, giving pPS1109. The 0.48-kb *Eco*RI-*Xba*I fragment from pTK4 carrying the *spoIIGB-spoIIIG* intergenic region was then isolated and inserted in pPS1109 cut with *Eco*RI and *Xba*I, giving pPS1110 (Fig. 1f). This plasmid cannot replicate autonomously in *B. subtilis* but can confer MLS resistance if it integrates into the chromosome at *spoIIIG*. If pPS1110 is first linearized with *Eco*RI, then MLS^r transformants carry a disrupted *spoIIIG* gene and are Spo$^-$ (Fig. 1f).

Introduction of the complete *spoIIIG* gene at *amyE*. Plasmid ptrpBGI was cleaved with *Hin*dIII and *Eco*RI, and the resulting small fragment was replaced with the *Eco*RI-*Hin*dIII polylinker of pUC12, giving pPS995. This plasmid was cleaved with *Xba*I (cleaving in the polylinker) and *Mlu*I. This removes essentially all of the *lacZ* coding region from pPS995. The remainder of the plasmid was ligated with the 1.5-kb *Xba*I-*Mlu*I fragment of pDG298, which contains all of the *spoIIIG* coding sequence but lacks the *spoIIIG*-specific promoter, giving pPS996 (Fig. 1g). This plasmid was linearized with *Nru*I and used to transform *B. subtilis* PS683 to a Cm^r Amy$^-$ phenotype by integration at the *amyE* locus (Fig. 1g) (strain PS1095). A second plasmid, pPS1093, was constructed by ligation of pPS1092 cut with *Sma*I with the 1.9-kb *Nae*I fragment from ptrpBGI carrying the *amyB* region of the *amyE* locus. The orientation of the *amyB* fragment in pPS1093 was established by digestion with *Sal*I. Plasmid pPS1093 was linearized with *Nru*I and used to transform PS1095 to an MLS^r CM^s Amy$^-$ phenotype. The resultant strain, PS1096, carries the intact *spoIIIG* plus the *spoIIIG*-specific promoter at the *amyE* locus (Fig. 1h). This complicated strategy was used because we were unable to stably clone the intact *spoIIIG* gene with its own promoter in *E. coli*. In strain PS1096, the direction of *ermC* transcription is the same as that of *spoIIIG*. A construct similar to PS1096 was also made in which the *ermC* gene was replaced by a *cat* gene (Fig. 1i). This was accomplished by transforming strain PS1096 with plasmid pPS939 cut with *Nru*I and *Sca*I and selecting for transformants which were Cm^r MLS^s. In this construct (strain PS1120) (Fig. 1i), the direction of *cat* transcription is away from *spoIIIG*.

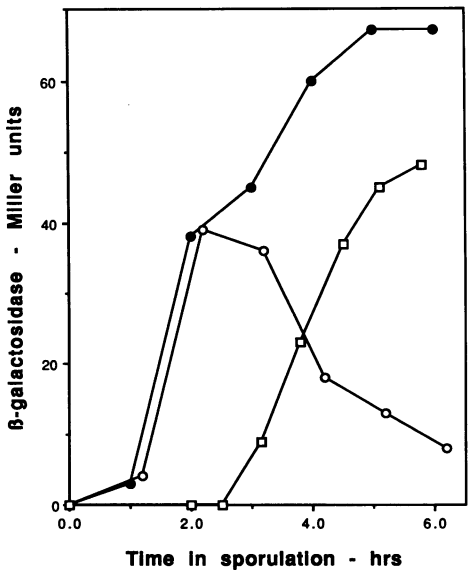

FIG. 2. Appearance of β-galactosidase during sporulation of strains carrying various *spoIIIG-lacZ* fusions. Strain PS832 (wild type) with plasmid pPS956 (●), pPS955 (○), or pPS896 (□) integrated at the *spoIIIG* locus (Fig. 1b to d) was sporulated, and β-galactosidase was assayed as described in Materials and Methods.

Integration of vectors into the *B. subtilis* chromosome. Competent cells of various *B. subtilis* strains were prepared and transformed with plasmids to chloramphenicol or MLS resistance as described previously (14). Transformation with pPS896, pPS955, and pPS956 used supercoiled plasmid, and integration into the chromosome was by a Campbell-type mechanism giving the chromosomal structure in the *spoIIG-spoIIIG* region as shown in Fig. 1b to d. This chromosomal structure was confirmed by appropriate restriction enzyme digestion and Southern blot analysis of chromosomal DNA. Note that in strains transformed with these three plasmids, the intact *spoIIIG* gene is ~7.5 kb downstream of the *spoIIG* operon.

Transformation of cells with pPS939, pPS1110, pPS996, and pPS1093 used plasmid linearized as described above and gave the chromosomal structure in either the *amyE* or *spoIIIG* region shown in Fig. 1e to i. Again these chromosomal arrangements were confirmed by Southern blot analyses.

RESULTS

Expression of *spoIIIG* during sporulation. Work in other laboratories has shown that transcription of the *spoIIG* operon begins at $\sim t_{0.5-1}$ and that there is at most a weak transcription terminator between *spoIIGB* and *spoIIIG* (8, 15). Consequently, mRNA potentially capable of directing σ^G synthesis should be present early in sporulation. Indeed, a promoterless *lacZ* gene integrated within the *spoIIIG* coding sequence (Fig. 1b) was expressed beginning at $t_{0.5-1}$, with a second rise in expression beginning at $\sim t_3$ (Fig. 2). A promoterless *lacZ* gene integrated in the intergenic region between *spoIIGB* and *spoIIIG* (Fig. 1c) was also expressed

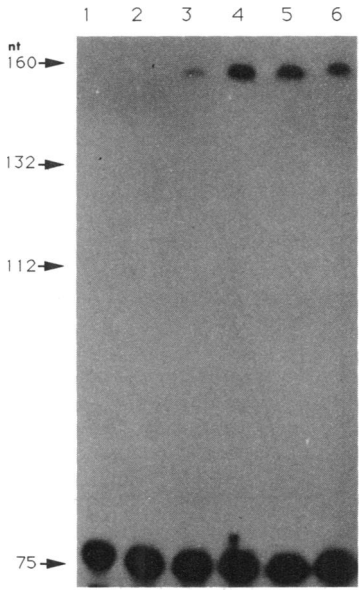

FIG. 3. Appearance of *spoIIIG*-specific mRNA during sporulation. Strain PS832 (wild type) was sporulated, cells were harvested at various times, and RNA was extracted and purified. Primer extension analysis was carried out by using the *Sca*I-*Hin*dIII primer to detect RNA with a 5′ terminus just upstream of *spoIIIG*. The times for harvest of cells for RNA isolation were as follows: lane 1, t_0; lane 2, $t_{1.5}$; lane 3, $t_{2.5}$; lane 4, $t_{3.5}$; lane 5, $t_{4.5}$; and lane 6, $t_{5.5}$. The numbered arrows adjacent to lane 1 denote the migration position of RNA size markers.

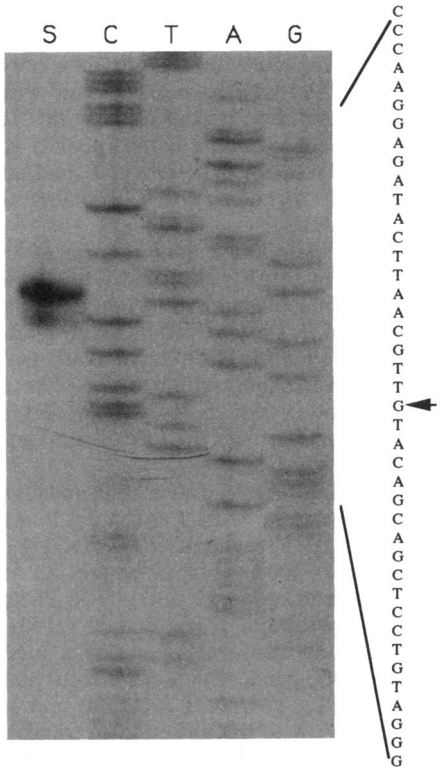

FIG. 4. Primer extension analysis of the 5′ end of *spoIIIG*-specific mRNA. RNA was isolated from sporulating cells of strain PS832 (wild type) at t_4 and analyzed by primer extension as described in Materials and Methods, using the *Sca*I-*Hin*dIII primer. The primer extension product (lane S) was run adjacent to the four lanes (C, T, A, and G) of sequencing reactions on the *spoIIIG* gene carried out by using the same primer. The arrow denotes the transcription start site.

early in sporulation (Fig. 2). However, with this construct the level of β-galactosidase fell after t_{2-3} (Fig. 2), presumably due to decreased expression plus degradation. These data suggest that the increased expression after t_3 of the promoterless *lacZ* gene integrated in the *spoIIIG* coding sequence was due to a second promoter in the *spoIIGB-spoIIIG* intergenic region (7, 15). The expression of a *spoIIIG-lacZ* translational fusion beginning only at $t_{2.5-3}$ (Fig. 2) is consistent with this suggestion. Primer extension analysis of RNA from sporulating cells demonstrated the appearance at $\sim t_{2.5}$ of an RNA species slightly smaller than 160 nt whose 5′ terminus was located between *spoIIGB* and *spoIIIG* (Fig. 1a and 3). Precise localization of the 5′ terminus of this *spoIIIG*-specific mRNA showed it to be at a G residue 29 nt before the *spoIIIG* translation start site (Fig. 4), which would give a 158-nt extended fragment with the *Sca*I-*Hin*dIII primer. The 5′ end of the *spoIIIG*-specific RNA determined by primer extension is almost identical to the 5′ end determined by nuclease protection (15).

Expression of *spoIIIG* in asporogenous mutants. One interpretation of the results given above is that *spoIIIG* is initially transcribed from the *spoIIG* promoter but that the *spoIIIG* portion of the resulting polycistronic mRNA is not translated until t_{2-3}, when a small amount of σ^G is made. This σ^G then directs transcription from a promoter just upstream of *spoIIIG*, producing a readily translated *spoIIIG*-specific mRNA (7, 25). However, it is possible that a transcription factor other than σ^G also becomes active at t_{2-3} and also directs *spoIIIG*-specific transcription; indeed, transcription from the *spoIIG* promoter may not be essential for initiation of *spoIIIG* expression. In support of the latter suggestion, in the transcriptional and translational *spoIIIG-lacZ* fusions (Fig. 1b to d), the wild-type *spoIIIG* gene is ~7.5 kb downstream from *spoIIG*, yet sporulation of these strains was identical to that of the wild type (data not shown). In addition, expression of the *spoIIIG-lacZ* translational fusion was not altered in a strain with a *spoIIIG* deletion mutation as found previously (5), although expression was abolished in *spoIIAC* and *spoIIIE* mutants (Fig. 5). RNA polymerase isolated from these latter two mutants at t_4 contained <10% of the wild-type level of σ^G (data not shown). Analysis of RNA isolated at t_4 from strains carrying various asporogenous mutations confirmed the results with the *lacZ* fusion, as the *spoIIIE* strain had no *spoIIIG*-specific mRNA (Fig. 6, lanes 1, 2, and 5), while the *spoIIIGΔ1* strain had the same amount as the wild type (Fig. 6, lanes 3 and 4). Note that these latter experiments were carried out with strains lacking *lacZ* fusions; the same results were obtained with the strains carrying the translational *spoIIIG-lacZ* fusion (data not shown). Similar results were also obtained when a synthetic single-stranded oligonucleotide was used in primer extension analyses; these latter reactions were clearly carried out in primer excess (data not shown).

Expression of *spoIIIG* at the *amyE* locus. The presence of *spoIIIG*-specific mRNA in a *spoIIIG* mutant suggested that σ^G itself is not required for *spoIIIG* expression. To examine *spoIIIG* expression without the complication of transcription from the *spoIIG* promoter and possible processing of a large transcript, a translational *spoIIIG-lacZ* fusion containing only ~200 nt of *spoIIIG* upstream sequence was integrated

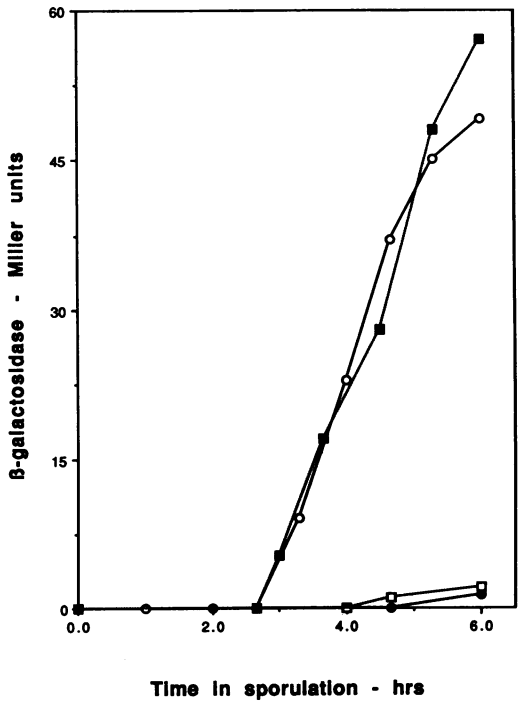

FIG. 5. Appearance of β-galactosidase during sporulation of strains carrying a translational *spoIIIG-lacZ* fusion integrated at the *spoIIIG* locus. Strains PS832 (wild type) (○), PS607 (*spoIIIE*) (●), PS683 (*spoIIIG*) (■), and PS1175 (*spoIIAC*) (□) with plasmid pPS896 integrated into the chromosome at the *spoIIIG* locus were sporulated, and β-galactosidase was assayed.

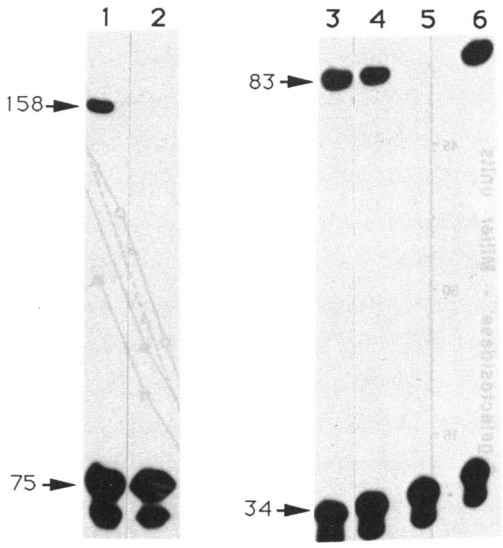

FIG. 6. Primer extension analysis of *spoIIIG*-specific mRNA from wild-type and mutant strains. RNA was isolated and purified from sporulating cells at t_4, and primer extension analysis was carried out by using the *ScaI-HindIII* (lanes 1 and 2) or *TaqI-ScaI* (lanes 3 to 6) primer as described in Materials and Methods. RNA was extracted from PS832 (wild type) (lanes 1 and 3), PS607 (*spoIIIE*) (lane 2 and 5), PS683 (*spoIIIG*) (lane 4), and PS607 (*spoIIIE*) carrying plasmid pPS939 integrated at the *amyE* locus (lane 6). The numbered arrows adjacent to the primers and extended transcripts in lanes 1 and 3 give the sizes in nucleotides determined with reference to the DNA sequence (7, 15) or a DNA sequencing ladder (Fig. 4 and data not shown).

into the *amyE* locus (Fig. 1e). This *spoIIIG-lacZ* fusion was expressed at the same time and to the same level as was the fusion at the *spoIIIG* locus, and expression was abolished by a *spoIIAC* mutation and reduced only ~40% in a *spoIIIG* background (Fig. 7). Surprisingly, essentially wild type levels of expression were seen in both *spoIIIE* and *spoIIIE spoIIIG* mutants (Fig. 7). Primer extension analysis of RNA from strains with the *spoIIIG-lacZ* fusion at the *amyE* locus showed that the 5′ end in the *spoIIIE* background was the same as that from the *spoIIIG* gene at the wild-type locus with use of either double-stranded fragments (Fig. 6, lanes 3 and 6) or a single-stranded oligonucleotide (data not shown) as a primer. Similar analyses showed that the 5′ end of *spoIIIG* mRNA from the *spoIIIG-lacZ* fusion at the *amyE* locus was also the same in *spoIIIG* and *spoIIIE spoIIIG* mutant backgrounds (data not shown). The difference in the effect of the *spoIIIE* mutation on expression of the *spoIIIG-lacZ* fusion at the *spoIIIG* and *amyE* loci was surprising. However, a number of other forespore-specific genes exhibit this same anomaly (27; see Discussion). It was previously reported that expression of a transcriptional *spoIIIG-lacZ* fusion integrated at the *amyE* locus is also independent of *spoIIIE* but is abolished by the *spoIIIGΔ1* mutation (7). We have confirmed this result but have also found that there is significant production of *spoIIIG*-specific mRNA from the *spoIIIG* promoter of this transcriptional fusion at the *amyE* locus (data not shown). At present we do not understand why this mRNA is not translated in the *spoIIIGΔ1* mutant.

The results noted above indicated that significant *spoIIIG* expression required neither σ^G itself nor read-through of transcription from *spoIIG*. If this is correct, then introduction of a wild-type *spoIIIG* gene at the *amyE* locus should suppress the asporogenous phenotype due to the *spoIIIGΔ1* mutation. This was the case, as both strains PS1096 and PS1120 (Fig. 1h and i) sporulated to ≥70% of wild-type levels, as measured by production of heat-resistant spores (heat-resistant forms per milliliter at t_{10}: PS832, 7.1×10^8; PS1096 and PS1120, 5×10^8) and the σ^G-dependent gene product, glucose dehydrogenase (data not shown). Analysis by Southern blotting as well as use of chromosomal DNA to transform Spo$^+$ strains demonstrated that both of these strains retained the *spoIIIGΔ1* mutation at the *spoIIIG* locus (data not shown).

Transcription of *spoIIIG* by $E\sigma^F$ and $E\sigma^G$. The results given above suggest that a factor other than σ^G can direct transcription of *spoIIIG*-specific mRNA. However, sequences just upstream of *spoIIIG* exhibit significant homology with the consensus −10 and −35 sequences recognized by $E\sigma^G$, and $E\sigma^G$ can initiate transcription in vitro on a *spoIIIG*-specific template (19). Furthermore, induction of σ^G synthesis in a *spoIIIGΔ1* strain carrying a translational *spoIIIG-lacZ* fusion at the *amyE* (data not shown) or *spoIIIG* (Fig. 8) locus resulted in induction of β-galactosidase synthesis roughly in parallel with that of glucose dehydrogenase, encoded by a known σ^G-dependent gene (26). Primer extension analysis of RNA from these induced cells showed that ~95% of the *spoIIIG* transcripts had initiated at the *spac* promoter, while ~5% had the 5′ terminus found in *spoIIIG*-specific mRNA from sporulating cells (data not shown). Thus, $E\sigma^G$ can initiate transcription of *spoIIIG*-specific mRNA in vivo. However, it has recently been shown that RNA polymerase containing the product of the *spoIIAC* gene, σ^F, can also initiate *spoIIIG* expression in vivo (21).

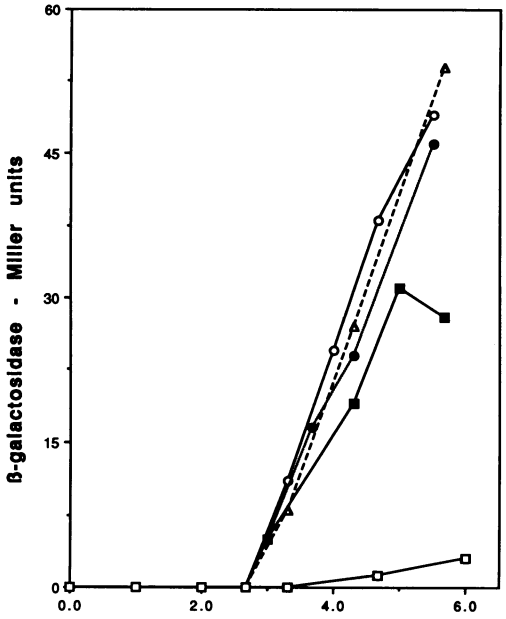

FIG. 7. Synthesis of β-galactosidase from the *spoIIIG-lacZ* fusion integrated at the *amyE* locus in various strains. Strains PS832 (wild type) (○), PS607 (*spoIIIE*) (●), PS683 (*spoIIIG*) (■), PS1128 (*spoIIIE spoIIIG*) (△), and PS1175 (*spoIIAC*) (□), all carrying plasmid pPS939 integrated into the chromosome at the *amyE* locus, were sporulated, and β-galactosidase was assayed.

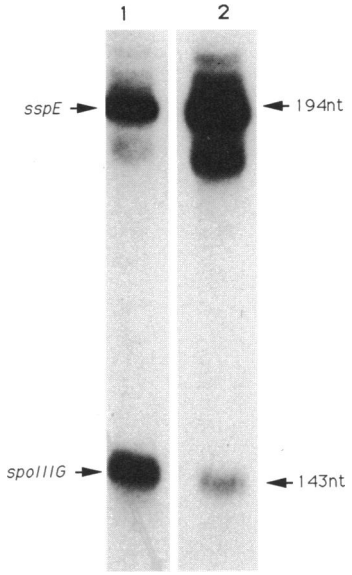

FIG. 9. In vitro transcription of *spoIIIG* by $E\sigma^F$ and $E\sigma^G$. A mixture of the *Bam*HI-cut *spoIIIG* template (1.6 μg) and *Eco*RI-cut *sspE* template (pPS591; 1 μg) was transcribed in vitro by $E\sigma^F$ or $E\sigma^G$, using [^{32}P]ATP, and transcripts were analyzed by electrophoresis on a 6% acrylamide gel. The sizes of the *sspE* and *spoIIIG* transcripts were determined with reference to RNA size markers as described in Materials and Methods. The size of the *spoIIIG* transcript is identical to that predicted from the sequence of pTK4 (8) if transcription in vitro begins at the same nucleotide as in vivo.

The level of *spoIIIG* expression reached upon induction of a single copy of the *spoIIAC* gene is similar to that achieved upon induction of *spoIIIG* in multicopy (Fig. 8) (21).

The transcription of *spoIIIG* in vivo by both $E\sigma^F$ and $E\sigma^G$ is possibly not surprising, since these two forms of RNA polymerase recognize similar promoter sequences in vitro (19). Indeed, both $E\sigma^F$ and $E\sigma^G$ initiated transcription on a *spoIIIG* template in vitro at identical start points, which were also identical to the 5′ terminus of *spoIIIG*-specific mRNA found in vivo (Fig. 9). However, quantitation of the activity of these two forms of RNA polymerase on a *spoIIIG* template relative to a known σ^G-dependent template (*sspE*), by cutting out and counting the transcripts, showed that $E\sigma^F$ had a 23-fold-higher preference than $E\sigma^G$ for the *spoIIIG* template relative to the *sspE* template (Fig. 9 and data not shown).

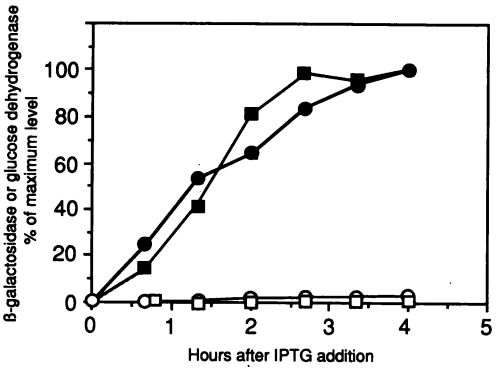

FIG. 8. Induction of *spoIIIG-lacZ* expression by $E\sigma^G$. Strain PS768 with plasmid pPS896 integrated into the chromosome at the *spoIIIG* locus was grown in 2×YT medium; at an optical density (OD) of 0.3 (t_0), one half of the culture was made 0.5 mM in IPTG to induce σ^G synthesis (induced) while the remaining half had no additions (uninduced). At various times, samples were harvested and analyzed for β-galactosidase and glucose dehydrogenase. Symbols: ●, β-galactosidase (induced); ○, β-galactosidase (uninduced); ■, glucose dehydrogenase (induced); □, glucose dehydrogenase (uninduced). The maximum β-galactosidase level reached was 285 Miller units; the maximum glucose dehydrogenase level was 130 nmol/min/OD_{600} of culture. At 4 h, the induced culture reached an OD_{600} of 3.1 and the uninduced culture reached an OD_{600} of 6.5.

DISCUSSION

Analysis of the expression of a promoterless *lacZ* gene integrated in the *spoIIIG* coding sequence indicates that *spoIIIG* is transcribed early in sporulation, undoubtedly from the *spoIIG* promoter. This was suggested earlier on the basis of nuclease protection analysis (15). However, the *spoIIIG* portion of this mRNA is translated poorly if at all. The delay in translation of *spoIIIG* mRNA is consistent with previous work which indicates that σ^G appears at $t_{2.5-3}$, only slightly before synthesis of σ^G-dependent gene products such as glucose dehydrogenase and the products of *ssp* genes (26). The reason for the lack of translation of the polycistronic *spoIIIG* mRNA has not been established. However, it was noted previously that this long mRNA could form a stem-loop structure in the region just prior to the *spoIIIG* translational start site which would sequester the gene's ribosome binding site (Fig. 10) (15). In contrast, the 5′ end of *spoIIIG*-specific mRNA cannot form this stem-loop structure (Fig. 10) and should be translated more efficiently.

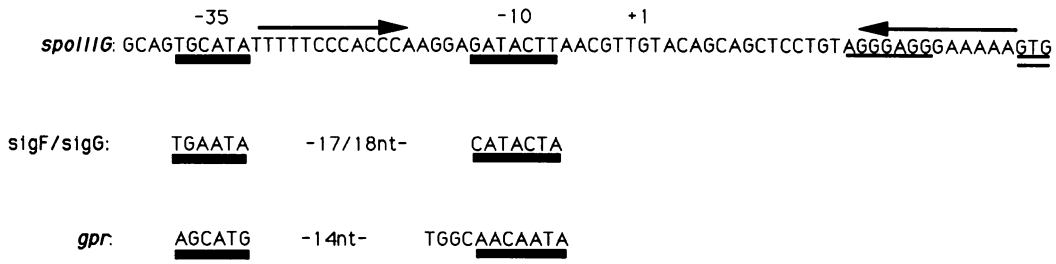

FIG. 10. Transcription start site of the *spoIIIG* gene. The *spoIIIG* sequence is taken from references 7 and 15. The *spoIIIG*-specific mRNA start site is denoted by the +1 over the G residue and is taken from Fig. 4. The opposed arrows above the sequence denote the regions in the polycistronic mRNA that can form the stem-loop which could block translation of this mRNA. The ribosome binding and translation start sites are singly and doubly underlined, respectively. The (σ^F/σ^G) (sigF/sigG) consensus sequences in the −10 and −35 regions are taken from reference 19. The *gpr* promoter sequence is taken from reference 29.

While the *spoIIIG*-specific mRNA formed at t_{2-3} of sporulation can be generated both in vitro and in vivo by $E\sigma^G$, it seems likely that under our sporulation conditions $E\sigma^G$ is at most a minor contributor in generating this mRNA species. This is shown most dramatically by the lack of effect of a *spoIIIG* mutation on *spoIIIG* expression, as measured by either a translational *spoIIIG-lacZ* fusion or the appearance of *spoIIIG*-specific mRNA. It could be argued that the appearance of *spoIIIG*-specific mRNA in a *spoIIIG* mutant is due to temporally determined processing of the polycistronic *spoIIIG* mRNA. However, if this is the case, then σ^G is not required for this processing and thus not for generation of *spoIIIG*-specific mRNA. Since the *spoIIIG* gene at the *amyE* locus is expressed with the correct 5′ terminus in a *spoIIIG* mutant, it seems more reasonable to propose that synthesis of *spoIIIG*-specific mRNA is due to initiation of transcription, presumably by $E\sigma^F$, at the 5′ end found in vivo. σ^F, the product of the *spoIIAC* gene, is made at $\sim t_{0.5}$ (30). The activity of $E\sigma^F$ has recently been shown to be regulated in some fashion by the other two products of the *spoIIA* operon, *spoIIAA* and *spoIIAB* (21); presumably this regulation allows $E\sigma^F$ action on the *spoIIIG* gene only in the forespore and only at t_{2-3}. This knowledge, plus the induction of *spoIIIG-lacZ* expression by σ^F synthesis in vegetative *B. subtilis* (21), the agreement between *spoIIIG*'s −10 and −35 sequences with those recognized by $E\sigma^F$, and the strong in vitro transcription of a *spoIIIG* template by $E\sigma^F$, lends support to our assignment of $E\sigma^F$ as the enzyme transcribing *spoIIIG*-specific mRNA. However, it is possible that other factors facilitate or modulate *spoIIIG*-specific transcription. Furthermore, while it appears clear that $E\sigma^F$ can efficiently initiate transcription of *spoIIIG*-specific mRNA in vivo, particularly in *spoIIIG* mutants, we have not yet proven that $E\sigma^F$ initiates the majority of *spoIIIG*-specific transcription during sporulation of wild-type cells.

It is striking that *spoIIIG* is a better template for $E\sigma^F$ in vitro than it is for $E\sigma^G$ relative to the activities of these holoenzymes on *sspE*. This suggests that the *spoIIIG* promoter may exhibit specific features of a good σ^F-dependent promoter. While the details of these features are not completely clear, a second largely σ^F-dependent gene termed *gpr* has recently been identified (29). Comparison of the *gpr* promoter sequence with that of *spoIIIG* reveals that both have purine residues at −13 and G residues at positions −15 and −16 (Fig. 10). That these residues may play a role in σ^F recognition has been recently demonstrated by introducing either a purine at −13 or two G residues at −15 and −16 in the *sspE* gene (normally σ^G dependent [26]) and showing that these changes allow this gene to be transcribed efficiently in vitro and in vivo by $E\sigma^F$ (3).

One rather anomalous result in this work was the different effects of a *spoIIIE* mutation on *spoIIIG* expression when the gene was at its own locus or at the *amyE* locus. We have found that expression of a number of other forespore genes (*gerA*, *gpr*, and *sspE*) becomes more or less independent of *spoIIIE* function when the genes are inserted at the *amyE* locus (28). While we have not yet proven that this *spoIIIE*-independent gene expression is confined to the forespore, if it is we have no explanation for this observation. However, we note that a dramatic condensation of the forespore chromosome takes place prior to t_3 of sporulation (22). Possibly this dramatic change in chromosome structure results in different effects on expression of genes in different regions of the chromosome.

Certainly one of the puzzling facets of *spoIIIG* regulation is why this gene is cotranscribed with *spoIIG* early in sporulation, yet the resulting polycistronic mRNA is not needed for *spoIIIG* expression. One possibility is that this is an anomaly of *B. subtilis* and that *spoIIIG* is separate from *spoIIG* in other *Bacillus* species. However, we have found that *spoIIIG* is adjacent to *spoIIG* in *B. cereus*, *B. megaterium*, and *B. stearothermophilus* (18). Clearly under our sporulation conditions *spoIIIG* need not be adjacent to *spoIIG* for efficient *spoIIIG* expression or sporulation, since introduction of up to 7.5 kb of DNA between *spoIIG* and *spoIIIG* does not affect sporulation, as found both by us and others (8). Similarly, a strain carrying a deletion in the *spoIIIG* gene adjacent to *spoIIG* plus a wild-type *spoIIIG* gene at *amyE* is Spo^+. Why then is this genetic and transcriptional organization of the *spoIIG* operon and *spoIIIG* maintained? Although we have no definitive answer to this question, one reason may be that there are two independent mechanisms for induction of synthesis of *spoIIIG*-specific mRNA. One is transcription by $E\sigma^F$ as we have found. The second may be the developmentally programmed initiation of translation of the polycistronic *spoIIG-spoIIIG* mRNA giving a small amount of σ^G, which then transcribes *spoIIIG*. Under our sporulation conditions $E\sigma^F$ appears to carry out the majority of *spoIIIG*-specific transcription, but laboratory sporulation conditions are far from the growth conditions found in the environment in which these regulatory mechanisms have been selected. Thus, it is possible that under different sporulation conditions, the $E\sigma^F$ pathway is a minor mechanism for *spoIIIG* expression and the $E\sigma^G$ pathway is the major one. The regulation of the activity or specificity of $E\sigma^F$ by the *spoIIAA* and *spoIIAB* gene products seems likely

to be sensitive to the physiological environment within the sporulating cell. It is easy to imagine that this environment could vary significantly in different sporulation conditions, thus allowing for different routes to σ^G synthesis. Since *spoIIAB* may also modulate the activity or specificity of σ^G (20), this adds another possible mechanism for switching between two pathways of *spoIIIG* expression.

ACKNOWLEDGMENTS

This work was supported by grant GM19698 from the National Institutes of Health.

We are grateful to J. Errington, P. Piggot, C. Moran, and R. Losick for gifts of strains and to R. Schmidt and R. Losick for helpful discussions and for communication of results prior to publication.

REFERENCES

1. **Antoniewski, C., B. Savelli, and P. Stragier.** 1990. The *spoIIJ* gene, which regulates early developmental steps in *Bacillus subtilis*, belongs to a class of environmentally responsive genes. J. Bacteriol. **172**:86–93.
2. **Connors, M. J., J. M. Mason, and P. Setlow.** 1986. Cloning and nucleotide sequence of genes for three small, acid-soluble proteins from *Bacillus subtilis* spores. J. Bacteriol. **166**:417–425.
3. **Fajardo-Cavazos, P., and P. Setlow.** Unpublished data.
4. **Ferrari, E., S. M. H. Howard, and J. A. Hoch.** 1985. Effect of sporulation mutations on subtilisin expression, assayed using a subtilisin–β-galactosidase gene fusion, p. 180–184. *In* J. A. Hoch and P. Setlow (ed.), Molecular biology of microbial differentiation. American Society for Microbiology, Washington, D.C.
5. **Foulger, D., and J. Errington.** 1989. The role of the sporulation gene *spoIIIE* in the regulation of prespore-specific gene expression in *Bacillus subtilis*. Mol. Microbiol. **3**:1247–1255.
6. **Goldrick, S., and P. Setlow.** 1983. Expression of a *Bacillus megaterium* sporulation-specific gene during sporulation of *Bacillus subtilis*. J. Bacteriol. **155**:1459–1462.
7. **Karmazyn-Campelli, C., C. Bonamy, B. Savelli, and P. Stragier.** 1989. Tandem genes encoding σ-factors for consecutive steps of development in *Bacillus subtilis*. Genes Dev. **3**:150–157.
8. **Kenney, T. J., and C. P. Moran, Jr.** 1987. Organization and regulation of an operon that encodes a sporulation-essential sigma factor in *Bacillus subtilis*. J. Bacteriol. **169**:3329–3339.
9. **Kunkel, B., R. Losick, and P. Stragier.** 1990. The *Bacillus subtilis* gene for the developmental transcription factor σ^K is generated by excision of a dispensable DNA element containing a sporulation recombinase gene. Genes Dev. **4**:525–535.
10. **Losick, R., and L. Kroos.** 1989. Dependence pathways for expression of genes involved in endospore formation in *Bacillus subtilis*, p. 223–242. *In* I. Smith, R. A. Slepecky, and P. Setlow (ed.), Regulation of procaryotic development. American Society for Microbiology, Washington, D.C.
11. **Mandelstam, J., and J. Errington.** 1987. Dependent sequences of gene expression controlling spore formation in *Bacillus subtilis*. Microbiol. Sci. **4**:238–244.
12. **Mason, J. M., P. Fajardo-Cavazos, and P. Setlow.** 1988. Levels of mRNAs which code for small, acid-soluble spore proteins and their *lacZ* fusions in sporulating cells of *Bacillus subtilis*. Nucleic Acids Res. **16**:6567–6583.
13. **Mason, J. M., R. H. Hackett, and P. Setlow.** 1988. Regulation of expression of genes coding for small, acid-soluble protein of *Bacillus subtilis* spores: studies using *lacZ* gene fusions. J. Bacteriol. **170**:239–244.
14. **Mason, J. M., and P. Setlow.** 1986. Essential role of small, acid-soluble spore proteins in resistance of *Bacillus subtilis* spores to UV light. J. Bacteriol. **167**:174–178.
15. **Masuda, E. S., H. Anaguchi, K. Yamada, and Y. Kobayash.** 1988. Two developmental genes encoding σ factor homologs are arranged in tandem in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **85**:7637–7641.
16. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–78.
17. **Moran, C. P., Jr.** 1989. Sigma factors and the regulation of transcription, p. 167–184. *In* I. Smith, R. A. Slepecky, and P. Setlow (ed.), Regulation of procaryotic development. American Society for Microbiology, Washington, D.C.
18. **Nicholson, W. L., and P. Setlow.** Unpublished data.
19. **Nicholson, W. L., D. Sun, B. Setlow, and P. Setlow.** 1989. Promoter specificity of a σ^G-containing RNA polymerase from sporulating cells of *Bacillus subtilis*: identification of a group of forespore-specific promoters. J. Bacteriol. **171**:2708–2718.
20. **Rather, P. N., R. Coppolecchia, H. DeGrazia, and C. P. Moran, Jr.** 1990. Negative regulator of σ^G-controlled gene expression in stationary-phase *Bacillus subtilis*. J. Bacteriol. **172**:709–715.
21. **Schmidt, R., P. Margolis, L. Duncan, R. Coppolecchia, C. P. Moran, Jr., and R. Losick.** 1990. Control of developmental transcription factor σ^F by sporulation regulatory proteins SpoIIAA and SpoIIAB in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **87**:9221–9225.
22. **Setlow, B., and P. Setlow.** Unpublished data.
23. **Setlow, P.** 1989. Forespore-specific genes of *Bacillus subtilis*: function and regulation of expression, p. 211–222. *In* I. Smith, R. A. Slepecky, and P. Setlow (ed.), Regulation of procaryotic development. American Society for Microbiology, Washington, D.C.
24. **Shimotsu, H., and D. J. Henner.** 1986. Construction of a single-copy integration vector and its use in analysis of regulation of the *trp* operon of *Bacillus subtilis*. Gene **43**:85–94.
25. **Stragier, P.** 1989. Temporal and spatial control of gene expression during sporulation: from facts to speculations, p. 243–254. *In* I. Smith, R. A. Slepecky, and P. Setlow (ed.), Regulation of procaryotic development. American Society for Microbiology, Washington, D.C.
26. **Sun, D., P. Stragier, and P. Setlow.** 1989. Identification of a new σ-factor involved in compartmentalized gene expression during sporulation of *Bacillus subtilis*. Genes Dev. **3**:141–149.
27. **Sun, D., M. Sussman, P. Fajardo-Cavazos, and P. Setlow.** Unpublished data.
28. **Sun, D., M. D. Sussman, and P. Setlow.** Unpublished data.
29. **Sussman, M. D., and P. Setlow.** 1991. Cloning, nucleotide sequence, and regulation of the *Bacillus subtilis gpr* gene, which codes for the protease that initiates degradation of small, acid-soluble, proteins during spore germination. J. Bacteriol. **173**:291–300.
30. **Wu, J.-J., M. G. Howard, and P. J. Piggot.** 1989. Regulation of transcription of the *Bacillus subtilis spoIIA* locus. J. Bacteriol. **171**:692–698.