JOURNAL OF BACTERIOLOGY, Mar. 1988, p. 1162–1167
0021-9193/88/031162-06$02.00/0

Vol. 170, No. 3

Transcriptional Regulation and Structure of the *Bacillus subtilis* Sporulation Locus *spoIIIC*

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Received 15 September 1987/Accepted 30 November 1987

The *spoIIIC* locus of *Bacillus subtilis* has been cloned from the lambda library of Ferrari et al. (E. Ferrari, D. J. Henner, and J. A. Hoch, J. Bacteriol. 146:430–432, 1981) by using as an assay transformation of the mutant allele *spoIIIC94* to the wild type. Regulation of the *spoIIIC* locus was studied by hybridization of cloned *spoIIIC* DNA to RNA pulse-labeled at various times during growth and sporulation. The relative rate of transcription of the *spoIIIC* locus was highest 3 h after the end of growth. The DNA sequence of the *spoIIIC* transcription unit indicated the coding capacity for a small protein (138 amino acids) having significant similarity with one domain of RNA polymerase sigma factors. Interruption of this coding sequence by an insertion mutation caused cells to become Spo$^-$.

The gram-positive soil bacterium *Bacillus subtilis* responds to nutrient deprivation by undergoing a series of metabolic and morphological changes that culminate in formation of a dormant endospore. These changes follow a temporal pattern that has been well defined. More than 50 genetic loci have been identified at which mutations (called *spo* mutations) block sporulation without inhibiting growth (22). Although the specific functions of these sporulation genes are generally unknown, the mutations cause blockage at identifiable morphological stages. Several of these sporulation genes have been isolated recently (1, 7, 8, 10, 16, 28, 35, 37). In some cases it has been possible to demonstrate specific transcription of particular sporulation genes at different times during sporulation (28, 32).

The study of other genes, which were isolated on the basis of their specific expression during sporulation, has provided strong evidence supporting the hypothesis (20, 21) that sets of genes are switched on or off at particular times during sporulation by sequential replacement of the sigma factor of RNA polymerase. This component of RNA polymerase is known to play a critical role in determining promoter specificity. Through the use of such cloned genes four minor vegetative forms of RNA polymerase, $E\sigma^{28}$, $E\sigma^{30}$, $E\sigma^{32}$, and $E\sigma^{37}$, and one sporulation-specific form, $E\sigma^{29}$, have been identified (5, 6, 12, 14, 15, 18). These studies demonstrated the specific transcriptional activities of these forms of RNA polymerase by using various genes as templates for in vitro experiments. These templates have not generally been genes whose products are clearly required for sporulation.

To test the generality of the sigma-replacement hypothesis for regulation of *spo* gene expression, we have been isolating additional genes whose products are required for sporulation. We previously reported the isolation and transcriptional analysis of the *spoIID* gene (28), and we report here initial studies on the cloned *spoIIIC* locus.

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MATERIALS AND METHODS

Cloning of the *spoIIIC* locus. The λ Charon 4A *B. subtilis* gene bank of Ferrari et al. (9) was found to transform strain 1S38 (*spoIIIC94 trpC2*; obtained from the Bacillus Genetic Stock Center) to Spo$^+$. A phage with this transforming activity was purified from the bank by successive testing of smaller and smaller pools of phages (3). Phage λ Ch2-93 contained six *Eco*RI fragments of *B. subtilis* DNA (4.7, 3.0, 1.7, 1.1, 0.9, and 0.7 kilobase pairs [kbp]), comprising a total of 12.1 kbp. Since the gene bank was constructed by partial *Eco*RI methylation of chromosomal DNA followed by complete *Eco*RI restriction, it is likely but not certain that these fragments are all contiguous in the chromosome. *Eco*RI fragments from λ Ch2-93 were ligated separately or in groups to the *Eco*RI site of vector pBR325 (4), inactivating the chloramphenicol resistance gene, and propagated in *Escherichia coli* MM294 (*endA hsdR thi pro*). Subclones were screened for retention of *spoIIIC*$^+$ transforming activity. Subclone pMR2 (Fig. 1) contained only two of the *Eco*RI fragments (two copies of the 1.7-kbp fragment and one copy of the 4.7-kbp fragment) from phage λ Ch2-93 and was able to transform strain 1S38 to Spo$^+$. Surprisingly, no subclones made from pMR2 (shown in Fig. 1), whether they contained either *Eco*RI fragment alone or regions overlapping the *Eco*RI junctions of pMR2, transformed strain 1S38 to Spo$^+$.

Pulse-labeling and isolation of RNA. *B. subtilis* SMY was pulse-labeled with [^{32}P]phosphate during vegetative growth in Sterlini-Mandelstam medium (34) supplemented with 0.5% glucose or at 1.5, 3, or 4 h ($T_{1.5}$, T_3, or T_4) after suspension from DSM medium (28) into Sterlini-Mandelstam medium. Three milliliters of cells was pulse-labeled for 5 min at 37°C with 3 to 6 mCi of carrier-free $^{32}PO_4$ (New England Nuclear Corp.). Incorporation was stopped by pouring the culture over frozen medium, and RNA was isolated and hybridized to a blot of DNA restriction fragments as described previously (3, 28).

Integrative plasmids. Plasmid pSGMU2 (11) is a derivative of plasmid pUC13 (24) into which a chloramphenicol resistance gene that can be expressed in *B. subtilis* has been inserted. Fragments of DNA from the *spoIIIC* region were subcloned into the polylinker site of pSGMU2 essentially as described by Messing (24). The resultant plasmids

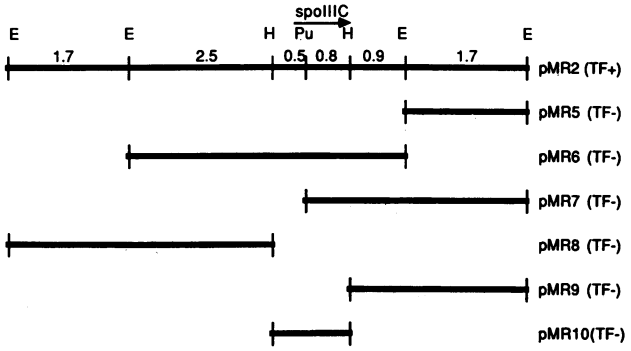


FIG. 1. Cloned *B. subtilis* DNA from the region of *spoIIIC*. Plasmid pMR2 was derived from λ Ch2-93, a Charon 4A derivative that carries 12.1 kbp of *B. subtilis* DNA (see Materials and Methods). Various regions of pMR2 were further isolated by subcloning or fragment elimination. Vector DNA (pBR325) is not shown. Plasmids retaining activity for transformation of strain 1S38 (*spoIIIC94*) to Spo$^+$ are designated TF$^+$. Restriction site abbreviations are as follows: E, *Eco*RI; H, *Hin*dIII; Pu, *Pvu*I.

(pSGMU54, pSGMU55, pSGMU56, pSGMU68, pSGMU69, pSGMU70) were transferred into competent cells of *B. subtilis* 168 (*trpC2*). Chloramphenicol-resistant transformants, which arose by integration of the plasmid at the *spoIIIC* locus, were tested for production of heat-resistant spores as described previously (11).

DNA sequencing. DNA fragments from plasmids pSGMU54, pSGMU55, and pSGMU56 were subcloned into phage M13mp18 or M13mp19 and sequenced by the chain-termination method of Sanger et al. (30, 31), except that gradient buffer gels and ^{35}S-labeled nucleotides were used (2). Some of the fragments for sequencing were obtained by limited digestion with nuclease *Bal* 31 as described by Maniatis et al. (23).

Construction of phage φ105J101. Plasmid pSGMU54 was linearized at its unique *Bam*HI site and cloned into phage vector φ105J23 (19) by transfection of protoplasts of *B. subtilis* 168 (7, 19) followed by selection for transduction of the same strain to resistance to chloramphenicol (5 μg/ml).

RESULTS

***spoIIIC94* mutation is a large deletion.** The inability of various subclones of pMR2 to give Spo$^+$ transformants by marker rescue of *spoIIIC94* (see Materials and Methods) would be understandable if *spoIIIC94* were a large deletion mutation or multiple point mutations. The original mutant carrying *spoIIIC94* was found among survivors of UV light treatment (17). To examine these possibilities, the restriction fragments in chromosomal DNA isolated from strain 1S38 and two different Spo$^+$ strains (all derived ultimately from strain 168) were compared by hybridization to radioactive cloned DNA. These experiments (data not shown) showed that the 1.7-kbp *Eco*RI fragment of pMR2 and pMR5 was present in all strains tested and that it was contained within a *Hin*dIII fragment of about 5 kbp. However, DNA of strain 1S38 (*spoIIIC94*), unlike that of the Spo$^+$ strains, did not hybridize at all to the 4.7-kbp *Eco*RI fragment present in pMR2 and pMR6. Thus it appears that the *spoIIIC94* mutation is a large deletion that removes part or all of one or more genes required for sporulation. Turner et al. (39) have shown that the 4.7-kbp *Eco*RI fragment, when inserted in phage φ105 DNA, can complement the *spoIIIC94* mutation.

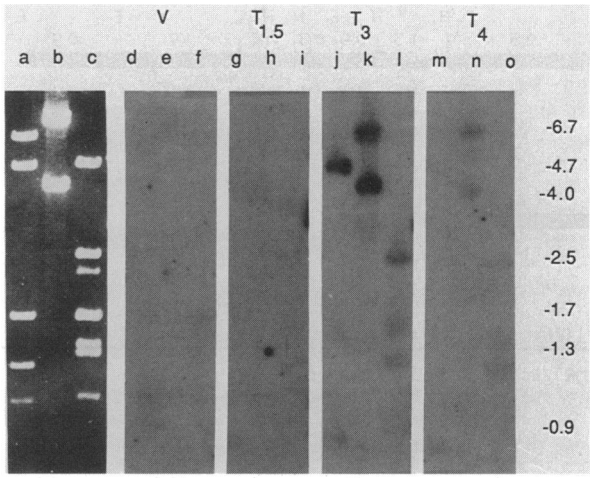
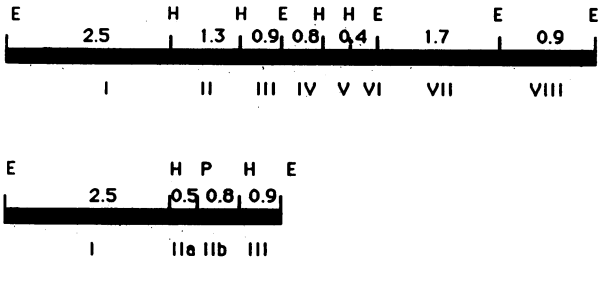


FIG. 2. Hybridization of cloned *spoIIIC* region restriction fragments to ^{32}P-pulse-labeled RNA. Plasmid pMR1 is a derivative of pBR325 into which four *Eco*RI fragments of *B. subtilis* DNA from λ Ch2-93 were cloned (see Materials and Methods). Plasmids pMR2 and pMR6 are shown in Fig. 1. RNA pulse-labeled at the times indicated during vegetative growth (*V*) and sporulation ($T_{1.5}$, T_3, T_4) was hybridized to restriction fragments of cloned DNA that had been separated by agarose gel electrophoresis (shown in photograph at left) and transferred to nitrocellulose. Lanes: a, d, g, j, and m, pMR1 cleaved with *Eco*RI; b, e, h, k, and n, pMR6 cleaved with *Pvu*I; c, f, i, l, and o, pMR2 cleaved with *Eco*RI and *Hin*dIII. Sizes of particular restriction fragments (in kilobase pairs) are shown on the right.

Temporal regulation of transcription of the *spoIIIC* region. Transcriptional regulation of the *spoIIIC* region was investigated by hybridization of nitrocellulose blots of restriction fragments of cloned DNA to RNA isolated from cells that had been pulse-labeled with $^{32}PO_4$ during growth or at various times during sporulation (Fig. 2 and 3). RNA hybridizing to both the 1.7- and the 4.7-kbp *Eco*RI fragments was preferentially synthesized during sporulation and was most prominent at 3 h after initiation of sporulation (T_3). Within the 4.7-kbp *Eco*RI fragment, a *Hin*dIII fragment of 1.3 kbp and its overlapping *Eco*RI-*Pvu*I subfragments were particularly strong in hybridization to RNA synthesized at T_3 and T_4 (Fig. 3). This pattern of regulation was not due to different levels of intactness of the RNA or to different specific activities. Blots were hybridized with equal amounts of radioactivity, DNA was in vast excess, and other genes exhibited different patterns of regulation when hybridized to the same RNA probes. For instance, DNA from the *citB* region hybridized only to RNA labeled during vegetative growth (data not shown); the *spoIID* gene and its neighboring gene *S* hybridized to RNA pulse-labeled at $T_{1.5}$, T_3, or T_4 (28).

Although these measurements of transcription in vivo indicate the existence within the 1.3-kbp *Hin*dIII fragment of a transcription unit active during sporulation, they do not show the orientation or limits of this transcription unit. Preliminary nuclease mapping experiments have shown that a transcript that first appears at T_2 and accumulates until at least T_4 lies within the 1.3-kbp *Hin*dIII fragment and crosses the internal *Pvu*I site in a left-to-right orientation. It is presumably this transcription unit that drives β-galactosidase expression in the *spoIIIC-lacZ* fusion strain constructed by Turner et al. (39). In such a strain, β-galactosidase activity first appears at T_3 and accumulates until T_5



DNA	RNA	Fragment I-III	IV-VI	VII	VIII
pMR1/EcoRI	VEG	-	-	-	+/-
	T1.5	-	-	-	+/-
	T3	++	-	+/-	+/-
	T4	-	-	-	+/-

		I	II	III	VII
pMR2/EcoRI, HindIII	VEG	-	-	-	-
	T1.5	-	-	-	-
	T3	+	+/-	+/-	+/-
	T4	+/-	+/-	-	+/-

		I,IIa	IIb,III
pMR6/PvuI	VEG	-	-
	T1.5	-	-
	T3	+++	++
	T4	+	+

FIG. 3. Transcription during growth and sporulation of the *spoIIIC* region. The data from Fig. 2 and similar experiments are diagrammed to show regions of sporulation-specific transcription. Restriction maps of the plasmids used are shown without vector DNA. Sizes are in kilobase pairs. The table indicates which fragments hybridized very strongly (+++), strongly (++), moderately (+), weakly (+/−), or not detectably (−) with RNA that was pulse-labeled with $^{32}PO_4$ during vegetative growth (*V*) or at 1.5, 3, or 4 h after resuspension in Sterlini-Mandelstam medium. The *spoIIIC* locus is thought to be located in fragment II (see the text).

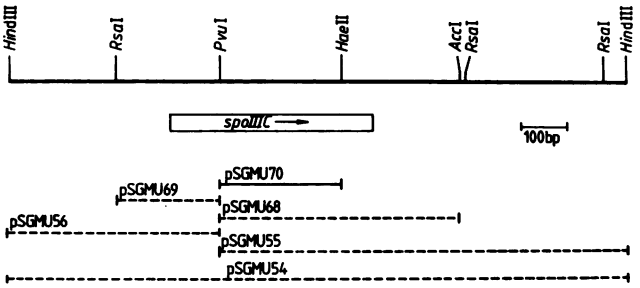


FIG. 4. Use of integrative plasmids to define the *spoIIIC* transcription unit. The upper part of the figure shows a partial restriction map of the 1.3-kbp *Hin*dIII fragment from the *spoIIIC* region. The open box shows the putative *spoIIIC* gene as determined by nucleotide sequencing (see the text and Fig. 5). The fragments of DNA subcloned in the integration plasmid pSGMU2, with plasmid designations, are shown at the bottom. The solid bar indicates that pSGMU70 gave a Spo$^-$ phenotype when integrated into the chromosome of *B. subtilis* strain 168; hence, both ends of this fragment lie within an essential part of the *spoIIIC* transcription unit. The other plasmids gave a Spo$^+$ phenotype (indicated by dotted lines) and therefore have at least one end outside the essential part of the transcription unit.

(39). The fusion was not expressed in several sporulation mutants blocked at stage 0 or stage II (39). For consistency and simplicity, we will refer to this transcription unit as *spoIIIC*, although it should be noted that the deletion mutation in strain 1S38 undoubtedly affects more than one sporulation gene.

Definition of the transcription unit with integrative plasmids. The use of integrative plasmids provides a useful genetic test for the extent of a transcription unit (27). Several segments of the 1.3-kbp *Hin*dIII fragment were subcloned in the integrative plasmid pSGMU2 (11). These plasmids replicate in *E. coli*, but not in *B. subtilis*; the chloramphenicol resistance that they code for can be expressed in *B. subtilis*, however, if the DNA integrates into the chromosome. Such integration is directed by the homology with the chromosome of the cloned DNA from the *spoIIIC* region and gives rise to Spo$^-$ transformants if the cloned segment is internal to a transcription unit whose product is essential for sporulation. Of the integrative plasmids shown in Fig. 4, only pSGMU70 caused the appearance of Spo$^-$ transformants. This is consistent with the *spoIIIC* transcription unit beginning between the leftmost *Rsa*I site and *Pvu*I site and ending before the *Acc*I site. As a further check on the location of the *spoIIIC* gene, the 1.3-kbp *Hin*dIII fragment of pSGMU54 was subcloned into the phage vector ϕ105J23 (19). When strain 618, a *spoIIIC* insertional mutant (39), was lysogenized with the new phage, designated ϕ105J101, a Spo$^+$ phenotype was restored. Since strain 618 is deleted for a DNA sequence that cannot be restored by recombination with ϕ105J101, we conclude that the 1.3-kbp *Hin*dIII fragment can complement a *spoIIIC* null mutation in *trans*, indicating that the *spoIIIC* gene is fully contained within this fragment.

Sequence of the *spoIIIC* region. Subfragments of the 1.3-kbp *Hin*dIII fragment were recloned in derivatives of phage M13 and sequenced by the chain termination method (30, 31) (Fig. 5). An open reading frame was seen (positions 364 to 777; 138 codons) in the orientation and location expected for the *spoIIIC* gene. We tentatively conclude that this open reading frame in fact represents the *spoIIIC* gene, since insertion mutations described above and previously (39) that interrupt this coding sequence cause a Spo$^-$ phenotype. There are three potential ATG translation initiation codons near the 5′ end of this open reading frame, of which we chose the first (positions 364 through 366) as the most likely start point. Although this region would have only moderate strength of interaction with the 3′ end of 16S r RNA (ΔG of −7 kcal [ca. −29.3 kJ] by the rules of Tinoco et al. [36]), it has stronger potential for interaction than do the sequences that precede the second and third ATG codons (ΔG of < -3 kcal [ca. −12.6 kJ]). The open reading frame is preceded by at least three potential promoter sites for the Eσ^{29} form of *B. subtilis* RNA polymerase. These promoters would be expected to activate transcription starting at position 247, 322, or 347. Preliminary in vitro transcription experiments indicate that this form of RNA polymerase does in fact transcribe the *spoIIIC* gene, initiating at approximately position 247. We have no evidence yet that this promoter is used in vivo.

A second, partially overlapping open reading frame was seen in the region upstream from *spoIIIC*, extending from the leftward *Hin*dIII site to position 379. The predicted protein would be at least 127 amino acids in length. There is no evidence at present that such a protein is actually made or that the region is transcribed in the appropriate orientation for this protein to be encoded. Another potential protein-coding sequence was located in the opposite orientation with

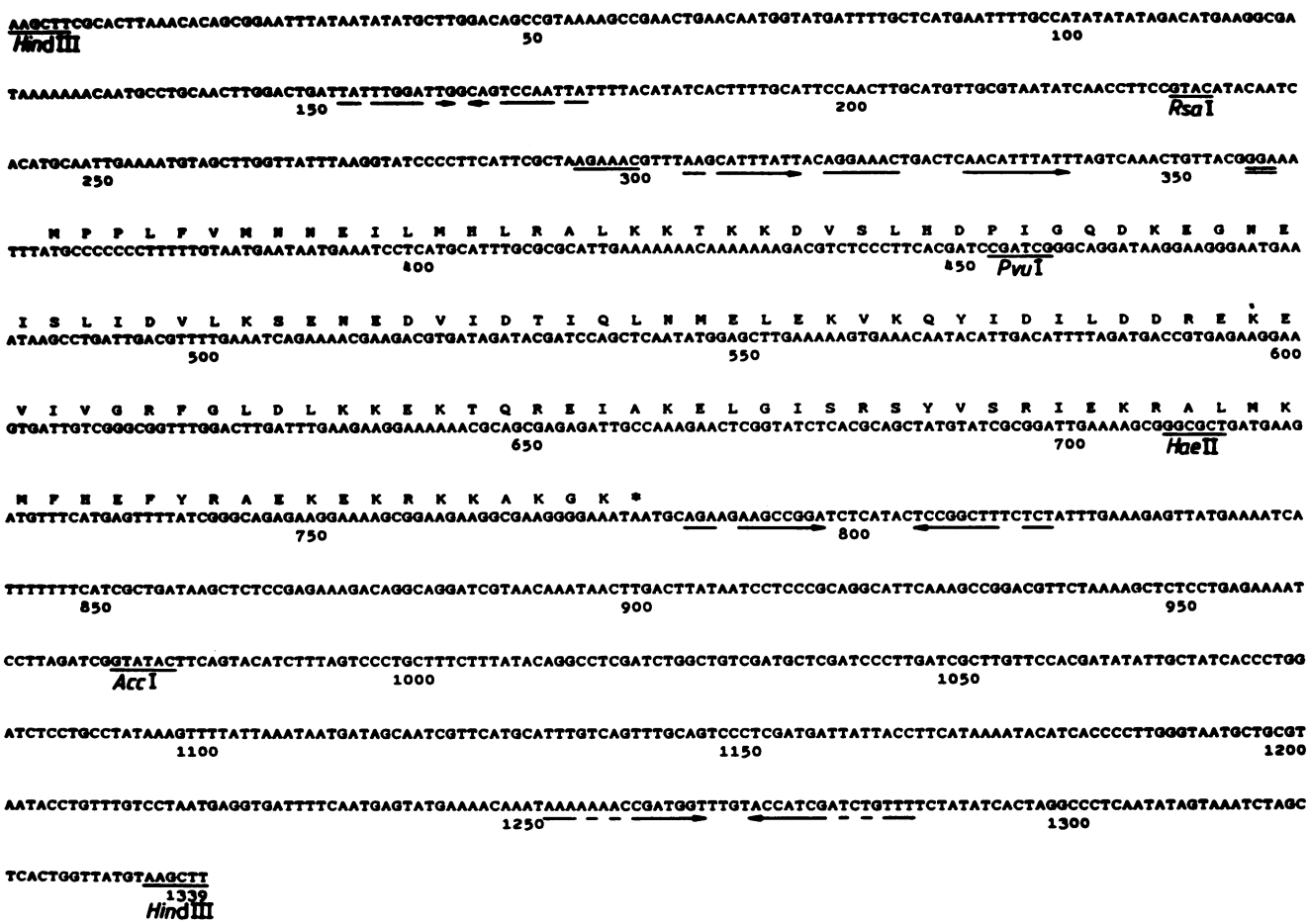


FIG. 5. Nucleotide sequence of the 1.3-kbp *Hin*dIII fragment. Restriction sites used for subcloning and sequencing are underlined. Above the putative *spoIIIC*-coding region is shown the predicted protein product with standard one-letter abbreviations for amino acids. A possible ribosome-binding site is double underlined. Arrows indicate the locations of regions of sequence containing extensive direct or inverted repeats.

respect to *spoIIIC*. It runs from positions 850 to 542, is preceded by a ribosome-binding site with a predicted free energy of −11 kcal (ca. −46 kJ), and could code for a protein of 103 amino acids. This open reading frame cannot represent the *spoIIIC* gene because the insert DNA in the integrative plasmid pSGMU70, which causes a Spo$^-$ phenotype, is not fully internal to this coding sequence. No other open reading frames capable of encoding proteins with more than 75 amino acid residues were found.

Comparison of the sequence of the predicted *spoIIIC* product with that of other proteins revealed significant similarity between a segment of the *spoIIIC* protein and the DNA-binding domains of sigma −29 (*sigE*) (Fig. 6) and other sigma factors (7a). This region of *spoIIIC* shows the helix-turn-helix motif characteristic of many DNA-binding proteins (13, 25), but the primary sequence is not similar to that of proteins other than sigma factors. A more detailed comparison of the predicted product of *spoIIIC* with other sigma factors is described elsewhere (7a).

DISCUSSION

The period of expression of the *spoIIIC* gene region corresponds roughly to the stage of arrest caused by the *spoIIIC94* mutation (there is some dispute as to whether this mutation causes blockage at stage III or stage IV [26]). Other sporulation genes whose time of expression in vivo has been studied have given mixed results in this regard. The *spoVG* gene is expressed very early during sporulation, but a defect in the gene product causes blockage only at stage V (29, 33). The *spoVA* locus is also transcribed before stage V (32). The *spoIIA* locus (32), the *spoIID* gene (28), and the *spoIIG* gene

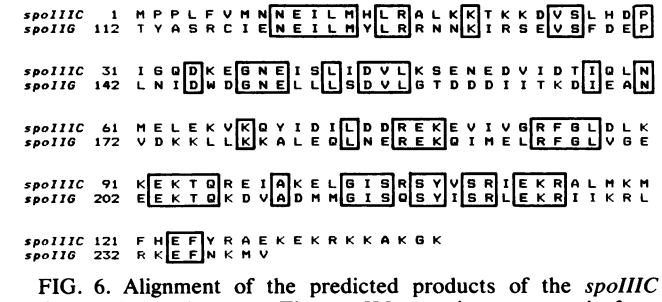


FIG. 6. Alignment of the predicted products of the *spoIIIC* and *spoIIG* (*sigE*) genes. The *spoIIG* protein sequence is from reference 35 and includes the N-terminal region removed in vivo from the precursor of σ^{29} (37). The standard one-letter codes for amino acid residues are used, and boxes indicate exact matches. (If very conservative amino acid replacements were also scored as identities, at least 24 additional positions would be in common.) The first residue in each line is numbered.

(38) are all transcribed preferentially at about the same stage of sporulation as the stage at which mutations at these loci cause blockage.

Further investigation of the cloned *spoIIIC* locus will help to validate the cascade model of regulation of sporulation gene expression by modification of the sigma factor component of RNA polymerase (20). At present, only one sporulation-specific form of RNA polymerase has been identified in *B. subtilis* (14). This form ($E\sigma^{29}$) appears at about T_1 but disappears after T_3. It transcribes in vitro the *spoIID* gene (28) and, in preliminary experiments, the *spoIIIC* region as well (Rong, unpublished data). However, since the initiation site in the latter region utilized in vitro by the $E\sigma^{29}$ form of RNA polymerase does not correspond to a site used in vivo and since the *spoIIIC* transcript accumulates in vivo at a time when the σ^{29} protein is greatly decreased in abundance (38), it is probable that one or more additional forms of RNA polymerase participate in transcription of the *spoIIIC* gene.

The DNA region that includes the *spoIIIC* transcription unit contains only one substantial open reading frame in the correct orientation and location. Although we have not shown directly that this open reading frame is used, it is interesting to speculate about its possible function. The sequence of the putative *spoIIIC* gene suggests that its predicted product might be capable of sequence-specific DNA binding; estimates of sequence similarity further suggest that its function is specifically related to that of RNA polymerase sigma factors (7a). If so, this protein probably would not act by itself as a sigma factor, since it is lacking the amino-terminal domain that has been suggested to give sigma factors the ability to interact with the core of RNA polymerase. It is possible that this protein would act in concert with a second polypeptide and that together they would provide a sigma factor-like function.

ACKNOWLEDGMENTS

We thank D. Bohannon, S. Brown, T. Henkin, K. Madden, M. Malamy, and F. Whipple for helpful discussions and review of the manuscript. We thank G. Roberts for excellent technical assistance and S. M. Turner for constructing some of the integration plasmids.

This work was supported by Public Health Service research grants GM19168 and GM30408 to A.L.S. and training grant T32-GM07310 from the National Institutes of Health. J.E. is the recipient of a Royal Society University Research Fellowship.

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