Identification of a new σ -factor involved in compartmentalized gene expression during sporulation of *Bacillus subtilis*

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During sporulation of *Bacillus subtilis*, two identical genomes segregate in two compartments, the forespore and mother cell. These genomes are expressed differentially, with some genes such as *sspE* turned on only in the forespore. In vitro transcription of *sspE* was obtained only with RNA polymerase extracted from sporulating cells. Fractionation of factors associated with this enzyme and reconstitution with core RNA polymerase from vegetative cells generated an enzyme accurately transcribing *sspE* in vitro and led to purification of a polypeptide with the amino-terminal sequence of the *spoIIIG* product. Inactivation of *spoIIIG* abolished expression of *sspE* and five other forespore-specific genes, whereas synthesis of the *spoIIIG* product in vegetative cells rapidly turned these genes on. Therefore, *spoIIIG* encodes a σ -factor, σ^G , which controls the expression of multiple genes in the forespore compartment.

[Key Words: *Bacillus subtilis*; *ssp* genes; σ -factors]

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Sporulation of *Bacillus subtilis* is a primitive example of compartmentalization of gene expression. The two identical genomes generated in the last round of DNA replication are segregated by an asymmetric septation in two unequal compartments that will become the forespore and the mother cell. These two cellular compartments progressively acquire different physiological properties, which are the reflection of differential gene expression. Ultimately, the mother cell lyses and releases into the medium a mature heat-resistant spore encased in a multilayered shell of coat proteins that have been synthesized in the mother cell (for review, see Losick et al. 1986).

Approximately 10–20% of the protein of *B. subtilis* spores is composed of a group of small, acid-soluble, proteins (termed SASP) located in the spore core and encoded by the *ssp* genes, five of which have been cloned and sequenced (for review, see Setlow 1988). All of these SASP are synthesized only during a defined period of sporulation, 3–5 hr after nutrient exhaustion (t_3 – t_5), and only within the forespore. Changes in the rates of SASP synthesis are paralleled by changes in levels of their mRNAs, a strong indication that the primary regulation of *ssp* gene expression is at the transcriptional level, such that *ssp* genes are transcribed only for a brief period in sporulation. The results of gene dosage experiments

using either *ssp* genes or *ssp*–*lacZ* gene fusion are also consistent with a major role for transcriptional control of the expression of these genes and further suggest that there is positive control of their expression (Mason et al. 1988a).

Previous work from a number of laboratories has indicated that a major mechanism for regulation of gene expression during sporulation in *B. subtilis* is the synthesis or activation of RNA polymerase-associated σ -factors with differing promoter specificities (Losick et al. 1986). To date, seven σ -factors (or σ -factor genes) have been identified in *B. subtilis*, of which two (σ^E and the *spoIIAC* product) are synthesized only during sporulation and undoubtedly play key roles in the alterations of the transcriptional pattern that takes place during this period of development (Losick et al. 1986). However, these two σ -factors do not seem to be involved directly in *ssp* gene transcription, and the results of Mason et al. (1988a) suggested that there might be an additional σ -factor that directs transcription of the *ssp* genes. In this paper we identify such a factor as the product of the *spoIIIG* gene and demonstrate its direct role in transcription of the *sspE* gene, as well as five other genes expressed only in the forespore. The *spoIIIG* gene is described and analyzed in the accompanying paper, Karmazyn-Campelli et al. (1989), which provides evidence that forespore-specific gene expression reflects compartmentalized synthesis of the *spoIIIG* gene product itself.

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Results

In vitro transcription of the *sspE* gene

Sporulating cell RNA polymerase purified through heparin–agarose gave a large amount of a 194-nucleotide transcript with *EcoRI*-cut *sspE* template (Fig. 1b). In contrast, heparin–agarose-purified enzyme from vegetative cells gave little, if any, transcript of this size with the same template (Fig. 1a). To prove that this 194-nucleotide transcript produced by the sporulating cell enzyme was from the *sspE* gene, we demonstrated further that (1) no transcript of this size was produced, when the *EcoRI*-cut vector was used as a template; (2) a shorter transcript of the expected size (cf. Fig. 2) was produced when *PvuII*-cut *sspE* template was used; (3) the 194-nucleotide transcript was also produced by sporulating cell enzyme with the *sspE* template cut with both *EcoRI* and *HincII*, as well as with the 0.8-kb *HindIII*–*EcoRI* *sspE* gene fragment alone (cf. Fig. 2) (data not shown).

The sizes of the runoff transcripts from the *sspE* template indicated that transcription *in vitro* initiated only 15 nucleotides preceding the gene's coding sequence (Fig. 2). This *in vitro* transcription start site has also been confirmed by dinucleotide priming experiments (W. Nicholson and P. Setlow, unpubl.). Strikingly, localization of the transcription start site of *sspE* mRNA made *in vivo* indicated that it is also only 15 nucleotides prior to the gene's coding sequence (Fig. 1c). Consequently, it appears that sporulating cell RNA polymerase can initiate *sspE* transcription accurately *in vitro*.

Figure 1. *In vitro* and *in vivo* transcription from the *sspE* gene. (Lanes *a*, *b*) Transcripts from the *EcoRI*-cut *sspE* template using heparin–agarose-purified RNA polymerase from vegetative cells (lane *a*) or from sporulating cells harvested at t_3 (lane *b*). Heparin was added in these assays. (Lane *c*) Nuclease-protected fragment obtained upon hybridization of the *sspE* antisense RNA probe with *sspE* mRNA made *in vivo*. The position of RNA size markers is indicated.

Purification of *sspE*-specific RNA polymerase

A likely explanation for the high activity of sporulating cell RNA polymerase on the *sspE* template *in vitro* is the presence of a new σ -factor in the sporulating cell enzyme. To determine when levels of this putative new σ -factor were highest, we determined the kinetics of appearance of *in vitro* transcription activity on an *sspE* template during growth and sporulation (Fig. 3). Although the RNA polymerase-specific activity with poly(dAT) as a template was relatively constant during growth and sporulation (data not shown), the activity on the *sspE* template increased >50-fold beginning around t_1 – t_2 and then decreased significantly (Fig. 3). The increase in RNA polymerase activity on an *sspE* template preceded the transcription of *ssp* genes *in vivo*, as measured by synthesis of β -galactosidase from an *sspE*–*lacZ* gene fusion (Fig. 3). The kinetics of appearance of the *sspE*-specific transcription activity during sporulation were identical in both the *spoIIIC94* strain (Fig. 3) and the parental strain (data not shown).

With data on the time of maximal *in vitro* transcription activity on the *sspE* template in hand, we grew a large amount of cells and harvested them at the peak of their *sspE* transcription activity. RNA polymerase was purified from 500 grams of cells by gradient elution from heparin–agarose and glycerol gradient centrifugation. Subsequent chromatography on DNA–cellulose gave two peaks of enzyme activity with poly(dAT); only the first peak had significant activity with the *sspE* template, although the elution profiles of enzyme activity with the different templates differed slightly (Fig. 4).

Reconstitution of an *sspE*-transcribing activity

Analysis of fractions across the DNA–cellulose column by SDS–polyacrylamide gel electrophoresis followed by silver staining did not allow an immediate correlation between a specific protein band and the *sspE*-transcription activity (data not shown). Therefore, peak fractions (see legend to Fig. 4) were pooled and concentrated prior to further analysis. Preliminary experiments with this pooled enzyme showed that when samples were run on SDS–polyacrylamide gels and proteins eluted and renatured, one or more proteins of apparent molecular mass of 30–40 kD could stimulate specific transcription of the *sspE* gene when added to core RNA polymerase from vegetative cells (data not shown). Consequently, ~2.5 mg of the pooled enzyme was run on a preparative SDS–polyacrylamide gel, and all proteins between 30 and 40 kD were eluted and concentrated. When these proteins were run on a high-resolution gel and the renaturation experiment was repeated, we found that either of two different protein bands (Fig. 5b,e) would promote *sspE* transcription. One of these bands (e) was a rather predominant protein but gave lower *sspE* transcription, whereas stronger *sspE* transcription was given by the much fainter band (b). Strikingly, the *in vitro* transcription start sites with either purified factor appeared identical to those with the unfractionated enzyme (control lane). These transcription assays were carried out

Figure 2. Restriction map of the *sspE* region and its transcripts. The DNA fragment is the 0.8-kb *Hind*III–*Eco*RI fragment cloned in pUC12, which contains 170 nucleotides from the amino-terminal portion of the *sspE* gene-coding sequence (hatched region) at the *Eco*RI end. The restriction map and DNA sequence is taken from Hackett and Setlow (1987). The doubly overlined sequence is the ribosome binding site; the singly overlined ATG is the translation start site. The vertical arrow above the G residue indicates the in vivo and in vitro transcription start site.

without heparin, which is probably the reason that some of the transcripts are smaller than 194 nucleotides.

A large aliquot of the partially purified factors was then run on a second high-resolution SDS–polyacrylamide gel, the proteins were transferred to Immobilon paper and stained lightly with Coomassie blue, and various stained bands were subjected to protein sequence analysis (Table 1). Band 3 was found to be the product of the *spoIIIG* gene and band e the product of the *spoIIAC* gene, whereas band d, which had no activity with the *sspE* template, was found to be σ^B . Although both the *spoIIAC* and *spoIIIG* gene products were predicted to be σ -factors, based on their amino acid sequence (Errington et al. 1985; Stragier 1986; Karmazyn-Campelli et al. 1989), these proteins have not been identified previously.

Involvement of the *spoIIIG* product in *sspE* expression in vivo

The *spoIIIG* product appeared to have the highest specific activity in directing *sspE* transcription in vitro. This finding, as well as data about *spoIIAC* and *spoIIIG* expression (see Discussion), strongly suggested that the *spoIIIG* product was of primary, if not sole, importance in *sspE* transcription in vivo. Consequently, we analyzed the effects of a *spoIIIG* deletion mutation on in vivo expression of *sspE*, as well as other forespore-specific genes.

Strikingly, the expression of the *sspE*–*lacZ* gene fusion was reduced to <2% of the wild-type level in a strain carrying the *spoIIIG* deletion, as was the expression of all other *ssp* genes tested (Table 2). The *spoIIIG* deletion mutation also abolished expression of two other

Figure 3. Appearance of *sspE* transcription activity during sporulation. Cells of the *spoIIIC94* strain carrying the *sspE*–*lacZ* fusion were grown, samples isolated, and enzyme purified through heparin–agarose, as described in Experimental procedures. Aliquots (50 μ g) of enzyme were assayed with the *Eco*RI-cut *sspE* template and the *sspE* transcript band was cut from the gel and counted. Samples of culture (1 ml) were also taken and assayed for β -galactosidase.

Figure 4. Chromatography of sporulating cell RNA polymerase on DNA-cellulose. Enzyme from the glycerol gradient step in the purification was applied to a column of DNA-cellulose, enzyme eluted as described in Experimental procedures, and assayed for activity with both poly(dAT) and the *EcoRI*-cut *sspE* template. In the latter assay, the *sspE* transcript band was cut from the gel and counted. Fractions 20–24 were pooled for further analysis.

genes that are turned on only in the forespore, *spoVA* and *gdh* (Table 2) (Fujita et al. 1977; Errington and Mandelstam 1986b).

As a complement to the experiments described above, we transformed the same strains with a plasmid carrying the *spoIIIG*-coding sequence under the control of an inducible promoter (see Experimental procedures). We then measured the synthesis of β -galactosidase or glu-

cose dehydrogenase following induction of synthesis of the *spoIIIG* product in vegetative cells. Although uninduced cells carrying the *sspE-lacZ* fusion made no β -galactosidase (Fig. 6a), synthesis of the *spoIIIG* product upon induction with IPTG resulted in rapid β -galactosidase accumulation (Fig. 6b) with the level of β -galactosidase achieved (in units per milliliter of culture) being ~60% of that in wild-type sporulating cells. Similar re-

Figure 5. *sspE* transcription activity of various proteins associated with RNA polymerase. Approximately 4 μ g of the 30,000- to 40,000-m.w. proteins associated with DNA-cellulose-purified RNA polymerase were denatured and run in adjacent lanes of a 15% polyacrylamide-SDS gel. One lane of the gel (horizontal gel) was silver stained (Merril et al. 1981), as was the edge of the second lane. Appropriate regions of the remainder of the unstained lane were then excised, and proteins eluted and renatured (Hager and Burgess 1980). The renatured proteins were added to core vegetative RNA polymerase, assayed for activity on *EcoRI*-cut *sspE* template without heparin, and the transcripts run on gel electrophoresis (vertical lanes). The control lane is sporulation RNA polymerase before removal of factors. Size markers are indicated.

Table 1. Automated sequenator analysis of protein bands from fractionated polymerase σ factor preparation

Cycle	Band b		Band d		Band e	
	Amino acid (pmole)	residue in <i>spoIIIG</i> gene product	amino acid (pmole)	residue in σ^B	Amino acid (pmole)	residue in <i>spoIIAC</i> gene product
1	Ser (43)	S	Thr (59)	T	Met (235)	M
2	Arg (9)	R	Gln (36)	Q	Asp (107)	D
3	Asn (18)	N	Pro (43)	P	Val (150)	V
4	Lys (19)	K	Ser (24)	S	Glu (127)	E
5	Val (21)	V	Lys (9)	K	Val (161)	V
6	Glu (16)	E	Thr (57)	T	Lys (200)	K
7	Ile (18)	I			Lys (168)	K
8	— ^a	C			Asn (78)	N
9	Gly (11)	G			Gly (51)	G
10	Val (12)	V			Lys (53)	K
11	Asp (12)	D			Asn (53)	N
12	Thr (12)	T			Ala (65)	A
13	Ser (10)	S			Gln (48)	Q
14	Lys (13)	K			Leu (62)	L
15					Lys (52)	K

Individual stained bands corresponding to those shown in Fig. 5 were subjected to automated protein sequenator analysis. The amino-terminal sequences of σ^B and the *spoIIAC* and *spoIIIG* gene products were taken from the work of Binnie et al. (1986); Fort and Piggot (1984); Karmazyn-Campelli et al. (1989).

^a This residue could not be identified.

sults were obtained when expression of the other forespore-specific genes was examined, 40–70% of the level of activity in wild-type sporulating cells being achieved after induction by IPTG; some slight differences in the kinetics of synthesis of different gene products following induction were also observed (data not shown). In all cases, induction of the *spoIIIG* product retarded cell growth significantly (see Fig. 6).

Discussion

The data reported in this paper allow identification of two new sporulation σ -factors as the products of the *spoIIAC* and *spoIIIG* genes, now designated, respectively, σ^F and σ^G , in accordance with the rules proposed by Losick et al. (1986). Although both of these polypeptides had been predicted to be σ -factors from similarities of their deduced amino-acid sequences with other σ -factors (Errington et al. 1985; Stragier 1986; Karmazyn-Campelli et al. 1989), they were defined initially only as the products of genes in which mutations blocked sporulation. Now, their copurification with RNA polymerase, as well as their effects on *sspE* transcription in vitro, indicate that they actually function as σ -factors.

σ^F is not involved in *sspE* transcription in vivo

The ability of σ^F to stimulate *sspE* transcription in vitro is somewhat surprising in light of what is known about its function and synthesis in vivo. It has been proposed that σ^F might also require the presence of the *spoIIAA* product for its action in vivo (Losick et al. 1986). This 13-kD polypeptide is much smaller than σ^F (Fort and Piggot 1984) and should not comigrate with it on SDS-polyacrylamide gels. Our results indicate that at least on

the *sspE* template, σ^F is sufficient to activate transcription in vitro and, furthermore, show that the σ^F polypeptide does not require amino-terminal processing to be active.

The time of synthesis of σ^F does not correlate with the activation of the *ssp* gene in vivo because the *spoIIA* operon is turned on around $t_{0.5}$ (Errington and Mandelstam 1986a), >2 hr before the *ssp* genes (Setlow 1988). However, it is consistent with the appearance of an activity transcribing *sspE* in vitro, well before synthesis of β -galactosidase from an *sspE-lacZ* gene fusion can be detected in vivo (Fig. 3). It is very likely that this initial transcription activity on *sspE* in vitro is due to σ^F , which does not recognize the *sspE* promoter in vivo. In this regard, we have recently found (D. Sun and P. Setlow, unpubl.) that RNA polymerase purified from the *spoIIIG* deletion mutant is still able to transcribe *sspE* in vitro (with at least 25% of the activity of the wild-type enzyme) despite the complete absence of expression of the *sspE-lacZ* fusion in that strain. Finally, it should be noted that mutations in many loci (*spoIIB*, *spoIIE*, *spoIIG*, *spoIIIA*) abolish *sspE* expression (Mason et al. 1988b) but do not interfere with the expression of the *spoIIA* operon (Errington and Mandelstam 1986a), indicating that σ^F synthesis cannot promote *sspE* expression in vivo by itself.

The simplest explanation for these discrepancies between the in vivo and in vitro results is to assume that the promoter recognition properties of σ^F and σ^G , the actual σ -factor for *sspE* (see below), are very similar. Such a possibility was predicted from sequence analysis of σ^F and σ^G , because they differ by only one amino acid in each of the two promoter recognition regions present in most σ -factors (Karmazyn-Campelli et al. 1989). Presumably, in vivo, such differences are sufficient to avoid

Table 2. Prevention of the expression of various forespore-specific genes by the *spoIIIG* deletion mutation

Gene tested	Maximal activity in <i>spoIIIG</i> Δ strain ^a
	Maximal activity in wild-type strain
<i>sspA-lacZ</i>	0.02
<i>sspB-lacZ</i>	0.01
<i>sspD-lacZ</i>	0.01
<i>sspE-lacZ</i>	0.01
<i>spoVA-lacZ</i>	0.04
<i>gdh</i>	0.03

^a Values are the ratios of the maximum activities obtained between t_4 and t_8 on growing cells in sporulation medium.

overlapping recognition between σ^F and σ^G , whereas in vitro, their specificities might be slightly relaxed. This situation is reminiscent of what has been described for the *spoVG* promoter, which can be recognized accurately in vitro by σ^B (formerly σ^{37}) (Johnson et al. 1983) but is actually under the control of σ^H , the *spoOH* product, in vivo (Zuber et al. 1988).

σ^G controls forespore-specific gene expression

A deletion mutation in *spoIIIG* abolishes expression of all the forespore-specific genes that we have tested, *sspA*, *sspB*, *sspD*, *sspE*, *spoVA*, and *gdh* (Table 2). Purified σ^G allows vegetative core RNA polymerase to initiate transcription accurately at the *sspE* promoter and with a higher efficiency than σ^F (Fig. 5), and this promoter is recognized efficiently by partially purified RNA polymerase from an *Escherichia coli* strain producing σ^G (D. Sun and P. Setlow, unpubl.). σ^G containing *B. subtilis* RNA polymerase also initiates transcription accurately in vitro at the *sspA*, *sspB*, *sspD*, and *gdh* promoters (W. Nicholson, B. Setlow, and P. Setlow, unpubl.). Tran-

scription of *sspE* and the five other forespore-specific genes that we have studied can be initiated in vivo during vegetative growth solely by inducing synthesis of σ^G (Fig. 6 and D. Sun and P. Setlow, unpubl.). Finally, transcription of *spoIIIG* starts at $t_{2.5}$, ~30 min before σ^G -inducible promoters are switched on (Karmazyn-Campelli et al. 1989).

Altogether, these data indicate that the *spoIIIG* product, σ^G , is necessary and apparently sufficient for initiation of transcription at six different forespore-specific promoters. However, it is certainly formally possible that an additional factor is needed for expression of some of these forespore-specific genes. An obvious candidate is the *spoIIIE* product, because mutations at the *spoIIIE* locus abolish expression of forespore-specific genes (Errington and Mandelstam 1986b; Mason et al. 1988b), whereas *spoIIIG* is expressed in these strains (Karmazyn-Campelli et al. 1989). However, σ^G -dependent genes can be switched on during vegetative growth in a *spoIIIE* mutant strain, as well as in wild-type cells (D. Sun and P. Setlow, unpubl.), indicating that the requirement for the *spoIIIE* product can be bypassed in these conditions.

Irrespective of the existence of auxiliary factors, all of our data point to the central role played by σ^G in forespore-specific gene expression. The minor differences that we observed in the kinetics of induction of σ^G -dependent promoters during vegetative growth may reflect a hierarchy of affinity of these promoters for σ^G . Examination of the sequences of a large number of these promoters reveals that they contain two highly conserved motifs in regions located 35 and 10 bp upstream of their known or postulated transcriptional start sites with the consensus sequence being TGAATA—17 or 18 bp—CATACTA (W. Nicholson, B. Setlow, D. Sun, and P. Setlow, unpubl.). It seems reasonable to postulate that these conserved nucleotides are the basis of recognition of these promoters by σ^G .

Figure 6. Induction of the *sspE-lacZ* fusion in vegetative cells by the *spoIIIG* product. The *B. subtilis* *spoIIIG*Δ1 *sspE-lacZ* strain carrying plasmid pDG298 was grown in 2 × YT medium to an OD of 0.25. At this time, IPTG was added to one half of the culture to 5 mM, and aliquots removed and assayed for β-galactosidase as a function of time.

Compartmentalization of gene expression during sporulation

Although the exact role of the *spoIIIE* product remains to be elucidated, σ^G appears to be one of the last links in the chain that leads to gene expression in the forespore. It has been shown that the great majority, if not all, of the *spoIIIG* product is itself localized in the forespore, as determined by localizing the β -galactosidase produced from the *spoIIIG-lacZ* transcriptional fusion (Karmazyn-Campelli et al. 1989). Thus, the control of forespore-specific gene expression must be exerted at the level of *spoIIIG* transcription, a process whose control remains to be elucidated.

Conversely, a mutation in *spoIIIG* does not block expression of the *spoIVCB* locus that is expressed specifically in the mother cell (Kunkel et al. 1988). It is tempting to speculate that other σ -factor(s) will be found to control specifically the expression of genes in the mother cell. Unraveling the mechanisms of their (compartmentalized?) synthesis in the light of those governing *spoIIIG* transcription should give us a useful model for tissue-specific gene expression during development.

Experimental procedures

Strains and plasmids

The parental strain used was *B. subtilis* 168. The *spoIIIC94* mutant strain was obtained from the Bacillus Genetic Stock Center, and the *spoIIIGA1* mutant strain is described in Karmazyn-Campelli et al. (1989). Strains carrying various *ssp-lacZ* gene fusions were constructed as described previously (Mason et al. 1988b). Phage Φ 105 carrying a *spoVA-lacZ* fusion was obtained from J. Errington and was transduced in various strains, as indicated by Errington and Mandelstam (1986b). Plasmid pDG298 carrying the *spoIIIG*-coding sequence under the control of the *spac* promoter was constructed as follows: The *StyI-BamHI* fragment carrying the first 166 codons of *spoIIIG* and 48 nucleotides of upstream flanking sequence (see Karmazyn-Campelli et al. 1989) was cloned after filling in the *StyI* site in the pJRD184 vector (Heusterspreute et al. 1985) between the *StuI* and *BamHI* sites (this cloning step introduced a *XbaI* site upstream of the *spoIIIG* DNA fragment); the *BamHI-RsaI* fragment carrying the last 95 codons of *spoIIIG* and 93 nucleotides of downstream flanking sequence was cloned in the Bluescript vector (Stratagene) between the *BamHI* and *EcoRV* sites (this cloning step introduced a *SalI* site downstream of the *spoIIIG* fragment); the complete *spoIIIG* coding sequence was reconstituted by cloning the *XbaI-BamHI* 'upstream fragment' with the *BamHI-SalI* 'downstream fragment' in the expression vector pDG148 (Stragier et al. 1988). pDG148 and pDG298 plasmids were amplified in *E. coli* and used to transform *B. subtilis* to kanamycin resistance (10 μ g/ml), as described previously (Mason et al. 1988b).

The *sspE* template used for in vitro transcription assays was a 0.8-kb *HindIII-EcoRI* fragment cloned in pUC12; for routine use, the plasmid was linearized with *EcoRI*. The same 0.8-kb *HindIII-EcoRI* fragment of the *sspE* gene was also cloned into plasmid pT7-1 (U.S. Biochemical Corporation), as described previously (Mason et al. 1988a). The orientation of the insert is such that an antisense *sspE* RNA probe is generated by T7 RNA polymerase action on *HindIII*-cut plasmid.

Enzyme assays

RNA polymerase assays with poly(dAT) as a template were carried out in 125 μ l containing 40 mM Tris-HCl (pH 8.0), 150 mM KCl, 10 mM MgCl₂, 0.4 mM DTT, 0.1 mM ATP and UTP, 0.025 μ Ci of ¹⁴C-labeled ATP, and 4 μ g of poly(dAT). The reaction was started by addition of enzyme to prewarmed reaction mixtures, and incubation was at 37°C. After 10 min, the reaction was stopped by the addition of 1 ml of cold 5% trichloroacetic acid, and the precipitate collected on a Whatman GF/C filter disk, rinsed three times with 5 ml of trichloroacetic acid and twice with 5 ml of ethanol, and the filter dried and counted in a scintillation counter. One unit of RNA polymerase was defined as the amount of enzyme that gave 1 nmole of ATP incorporation in 10 min. The RNA polymerase runoff transcription assay using the *sspE* template was carried out in 125 μ l containing 40 mM Tris-HCl (pH 8.0), 50 mM KCl, 10 mM MgCl₂, 0.4 mM DTT, 0.2 mM CTP, GTP, and UTP, 0.18 μ M unlabeled ATP, 2 μ Ci [α -³²P]ATP, and 2 μ g of *EcoRI*-cut *sspE* template. Reactions were started by addition of enzyme to prewarmed reaction mixtures, incubated at 37°C for 5 min; 5 μ l of 5 mM unlabeled ATP was added, and incubation continued for an additional 5 min. Reactions were stopped by mixture with an equal volume of phenol/chloroform (1 : 1). After extraction, nucleic acids were precipitated with ethanol, the precipitates were dissolved in gel loading buffer and run on a 6% polyacrylamide-7 M urea gel, and the gel was autoradiographed. For precise quantitation in this assay, the *sspE* transcript band was cut from the gel and counted in a scintillation counter. In some experiments, heparin was added 2 min after the start of the reaction to reduce reinitiation and prevent nuclease digestion. To test the activity of reconstituted σ -factors, 0.05 unit of vegetative core RNA polymerase plus 15 μ l of renatured σ -factors were added to the reaction mixture without nucleoside triphosphates. After 5 min at 4°C, the mixture was prewarmed to 37°C for 30 sec and the reaction started by adding the nucleoside triphosphates. Cells to be assayed for β -galactosidase or glucose dehydrogenase were harvested, permeabilized with lysozyme, and assayed as described previously (Mason et al. 1988a).

Appearance of *sspE* transcription activity during sporulation

To determine the time course of appearance of *sspE* transcription activity during sporulation, wild-type cells were grown in 2 $\times$ SG medium (Goldrick and Setlow 1983) at 37°C in an 11-liter fermentor. Samples (250 ml) were harvested at various times, washed extensively to remove extracellular proteases (Nakayama et al. 1977), and suspended in cold buffer A [10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 0.3 mM DTT, 7.5% glycerol, and 2 mM PMSF]. Cells were broken by three passes through a French press at 12,000–14,000 psi. After high-speed centrifugation, the supernatant fluid was dialyzed against buffer A. The dialyzed extract was absorbed to a column of heparin-agarose (Davison et al. 1979), equilibrated with buffer A; the column was washed and enzyme eluted with buffer A containing 1 M NaCl. Fractions with high activity with poly(dAT) were pooled, dialyzed against storage buffer [10 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 0.1 mM EDTA, 0.3 mM DTT, 100 mM KCl, and 50% glycerol], and stored at -20°C.

RNA polymerase purification

Wild-type cells were grown to mid-log phase in 2 $\times$ YT medium (Loshon et al. 1986), and RNA polymerase was purified through the DNA-cellulose column step using published procedures (Shorenstein and Losick 1973). The vegetative core RNA poly-

merase was prepared by chromatography on phosphocellulose (Losick et al. 1970).

RNA polymerase with high activity on the *sspE* template was purified from sporulating cells of the *spoIIIC94* mutant strain carrying the *sspE-lacZ* gene fusion. This strain was used because the *sspE-lacZ* fusion was expressed to a higher level in that strain than in wild-type cells (Mason et al. 1988b), and the cells were easier to break. Cells were grown at 37°C in 2 × SG medium, and 1-ml aliquots were taken for assay of β-galactosidase. These assays were carried out as described previously (Mason et al. 1988b), except that lysozyme was at 250 μg/ml and the assay was only for 10 min. When the β-galactosidase activity reached ~20% of its maximum value, cells were harvested, washed as described above, and frozen. Frozen cells (500 grams) were suspended in 1 liter of cold buffer A, broken as described above, and RNA polymerase purified from the high-speed supernatant fluid by gradient elution from heparin-agarose, followed by preparative glycerol gradient centrifugation (Davison et al. 1979).

The RNA polymerase activities on poly(dAT) and the *sspE* template copurified throughout these steps, which yielded ~40 mg of protein. This enzyme was dialyzed overnight against buffer B [50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.3 mM DTT, 0.5 mM PMSF, and 20% glycerol] containing 100 mM KCl. The dialyzed enzyme was loaded on a column of calf-thymus DNA-cellulose (Alberts and Herrick 1971) equilibrated with buffer B containing 100 mM KCl, the column was washed and enzyme eluted with a linear gradient of 0.3–1.1 M KCl in buffer B. Fractions (4 ml) were collected, MgCl₂ added immediately to 10 mM, and fractions were assayed for RNA polymerase activity with poly(dAT) and the *sspE* template. Fractions with high activity on *sspE* DNA were pooled, dialyzed overnight against storage buffer, and stored at –20°C.

Identification of factors stimulating *sspE* transcription

DNA-cellulose-purified enzyme (2.5 mg) was concentrated with a 30,000 m.w. cutoff Centricon concentrator (Amicon), denatured, and electrophoresed through a 10% polyacrylamide–SDS gel (Laemmli 1970). A slice encompassing the apparent 30,000 to 40,000 m.w. range was cut out, and proteins were recovered by electroelution, concentrated, and stored at –80°C. For protein sequence analyses, ~50 μg of these concentrated factors were denatured, run on a 15% polyacrylamide–SDS gel, electroblotted to a sheet of polyvinylidene difluoride paper (Immobilon, Millipore Corp.), and lightly stained with Coomassie blue using a published procedure (Matsuidara 1987). Appropriate stained protein bands were cut out and subjected to sequence analysis on an Applied Biosystems model 470A gas-phase sequencer equipped with a model 120A PTH analyzer.

Determination of *in vivo* *sspE* mRNA transcription start site

RNA from sporulating cells containing high levels of *sspE* mRNA was isolated as described previously (Hackett and Setlow 1987). The transcription start site for *sspE* mRNA was determined by ribonuclease protection essentially as described by Zinn et al. (1983). The *sspE* antisense RNA probe was synthesized by T7 RNA polymerase using [α-³²P]ATP and the *Hind*III-cut pT7-1 vector carrying the *sspE* gene described above (Mason et al. 1988a). RNA markers were made by runoff transcription with T7 RNA polymerase, using fragments of known DNA sequence cloned in plasmid pT7-1.

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