Genetic Evidence for Interaction of $\sigma^E$ with the spoIID Promoter in Bacillus subtilis

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During sporulation in Bacillus subtilis, new RNA polymerase sigma factors are produced. These sigma factors direct the transcription of genes that are required for this cellular differentiation. In order to determine the role of each sigma factor in this process, it is necessary to know which promoters are recognized by each sigma factor. The spoIID gene product plays an important role in the establishment of mother cell-specific gene expression during sporulation. We found that substitution of an alanine at position 124 of the sporulation-specific sigma factor $\sigma^E$ suppressed the effect of a single-base-pair transition at position $-13$ of the spoIID promoter. This alanine substitution in $\sigma^E$ did not suppress the effect of a transversion at position $-12$ of the spoIID promoter. The allele specificity of the interaction between $\sigma^E$ and the spoIID promoter is strong evidence that $\sigma^E$ directs transcription from the spoIID promoter during sporulation. Position 124 in $\sigma^E$ is located within a region that is highly conserved among the regions in other sigma factors that probably interact with the $-10$ regions of their cognate promoters.

The sigma subunit of bacterial RNA polymerase determines the specificity of promoter utilization. In Bacillus subtilis, new sigma factors are produced during sporulation, resulting in the transcription of genes that are required for this cellular differentiation (reviewed in references 19 and 27). The production of these sigma factors appears to play an important role in regulating gene expression during sporulation. However, since several sigma factors are present simultaneously during sporulation, it has been difficult to determine which sigma factor is responsible for the transcription of each sporulation-essential gene. Therefore, the exact role of each sigma factor remains unclear.

Recently, a genetic approach has been used to provide compelling evidence that $\sigma^A$ (11) and $\sigma^H$ (1, 32) interact directly with specific promoters during sporulation. These studies and those on $\sigma^70$ in Escherichia coli (5, 24, 29) support the model that sigma factors govern the specificity of promoter utilization by making sequence-specific contacts at two regions of their cognate promoters, the $-10$ and $-35$ regions. In these studies, single-amino-acid substitutions in the sigma factors were found to suppress the effects of specific single-base-pair substitutions in promoters. For example, substitution of threonine at position 100 of $\sigma^H$ suppressed the effect of a base pair substitution at position $-13$ of the spoVG promoter but not the effects of substitutions at other positions in the spoVG promoter (32). Substitution of alanine at position 96 of $\sigma^H$ partially suppressed the effect of a substitution at position $-12$ of the spoVG promoter (1). The position-specific suppression of promoter mutations by the amino acid substitutions at positions 96 and 100 of $\sigma^H$ is strong evidence that this region of the sigma factor directly contacts the $-10$ region of the spoVG promoter. These results and those of similar experiments with $\sigma^70$ from E. coli (5, 24) inspired experiments with $\sigma^A$ in which substitution of arginine at position 196 of $\sigma^A$ was shown to suppress the effects of a base pair substitution at position $-11$ in the spoIIIG promoter (11). The allele specificity of this suppression was taken as strong evidence that $\sigma^A$ interacts with the spoIIIG promoter during sporulation. Subsequently, suppression experiments have been used to demonstrate interaction of $\sigma^A$ with three additional promoters in B. subtilis (9, 31). These results provide important clues concerning the molecular interactions of sigma factors with their cognate promoters; however, the more significant implication for the study of sporulation probably is that these altered sigma factors provide tools that can be used to show which sigma factors interact with which promoters during sporulation.

During endospore development the cell is divided into two compartments in which differential gene expression results in different developmental fates. The mother cell becomes a terminally differentiated cell that provides specialized products to the developing endospore, while the forespore compartment develops into the endospore, which will remain dormant until germination. The spoIID gene product is a DNA-binding protein that is necessary for mother cell-specific gene expression (12, 14). Analysis of spoIID-lacZ fusions demonstrated that spoIID expression begins 2 h after the onset of sporulation and that this expression is dependent upon the product of sigE (14, 25). sigE encodes the RNA polymerase sigma factor $\sigma^E$, which is produced about 2 h after the onset of sporulation. The temporal pattern of spoIID expression as well as its dependence on sigE and other sporulation genes has been used to suggest that spoIID transcription is directed by $\sigma^E$ (14, 25). In an alternative model, the dependency of spoIID expression on sigE could result because $\sigma^E$ directs the expression of an unknown factor that is required for spoIID expression. In this model, $\sigma^E$ does not interact directly with the spoIID promoter. We wished to determine whether genetic suppression experiments similar to those described above for $\sigma^H$ and $\sigma^A$ could be used to demonstrate that $\sigma^E$ interacts with the spoIID promoter during sporulation. Our strategy required localization of the spoIID promoter, characterization of single-base-pair substitutions in the spoIID promoter, and isolation of a mutant allele of sigE which resulted in a single-amino-acid substitution in $\sigma^E$ that suppressed the effect of a base pair substitution in the spoIID promoter.

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MATERIALS AND METHODS

RNA isolation and primer extension analysis. _B. subtilis_ JH642 was grown in DS medium (23) with vigorous shaking at 37°C. When the culture reached an OD₆₀₀ of 0.3, it was diluted fivefold in DS medium and incubation at 37°C was continued. Samples (100 ml) were harvested during sporulation, and RNA was isolated as previously described (8). The procedure for primer extension analysis has been described previously (8). The sequences of the oligonucleotide that was used to prime the DNA synthesis was 5'-GCTTCTTCGT TGTATGG-3'.

Cloning and mutagenesis of the _spolIID_ promoter. A 130-bp _XmnI-DraI_ fragment from pBK39 (14) containing the _spolIID_ promoter region from positions −72 to +63 was cloned into pUC18 (30) at the _HincII_ site, creating pUC18-IIID. The orientation of the _spolIID_ promoter with respect to the _HincII_ site was determined by restriction endonuclease mapping and confirmed by nucleotide sequencing. The 160-bp _HindIII-BamHI_ fragment from pUC18-IIID was cloned between the _HindIII_ and _BamHI_ sites of both M13mp18 and M13mp19, generating mp18-IIID and mp19-IIID, respectively.

Single-nucleotide substitutions in the −10 region of the _spolIID_ promoter were constructed by oligonucleotide-directed mutagenesis by the procedure of Kunkel (15) as described previously (22). These sequences of the oligonucleotide used to prime the DNAsynthesis was 5'-GCTTCTTCGT TGTATGG-3'. The sequences used to generate these mutants were as follows: 5'-GAATGCTATTACACTG-3' was used to construct mp18IIID12T, and 5'-AGAATGCTATTACACTG-3' was used to construct mp18IIID13G. The replicative form (RF) of each mp18IIID derivative was prepared, and the nucleotide sequence was determined to confirm that the correct mutations were constructed.

Transfer of _spolIID-lacZ_ transcriptional fusions to SPB. Promoter-lacZ fusions were constructed by excising the 160-bp _HindIII-BamHI_ fragment from the RF of each mp18-IIID derivative and cloning each between the _HindIII_ and _BamHI_ sites of pZAB/HNB (9), which contains a promoterless derivative of the _E. coli_ lacZ gene. Nucleotide sequencing of the promoter fusions was used to confirm that the proper constructions were made. After the p2ΔB derivatives were linearized with _SsrI_, they were used to transform strain ZB307A (20) to chloramphenicol resistance. Homology between the pZAB derivatives and the prophage SPc2de12::_Tn917_ pSK10Δ6 in the _B. subtilis_ chromosome allowed a double crossover event to occur, generating strains in which the _spolIID-lacZ_ fusion is on the SPB prophage and is now selectable by resistance to chloramphenicol. The SPB phage was induced from the chromosome of each strain by heat as described previously (22), generating the following phage lysates: SPBIIID-lacZ, SPBIIID12T-lacZ, and SPBIIID13G-lacZ.

Cloning and mutagenesis of _sigE_. A 1.1-kb _PstI_ fragment containing _sigE_ was cloned into the _PstI_ site of pJ8900. pJ8900 (6) is an expression plasmid which contains the _pUC1_ origin for replication in _E. coli_, the gene encoding bleomycin resistance, and the _pUB110_ origin for replication in _B. subtilis_. The _lac_ promoter (Plac) in pJ8900 was changed to the consensus sequence for a sigma A promoter (Plac₆₉₃) (9) by oligonucleotide-directed mutagenesis, generating pJ8903.

To obtain single-stranded phagemid DNA for mutagenesis of the _sigE_ gene, _E. coli_ RY2504 containing pJ8903 was infected with the helper phage M13K07 and grown in the presence of kanamycin at 70 μg/ml, bleomycin at 7 μg/ml, and uridine at 0.25 μg/ml. The mutation in _sigE_ was constructed by oligonucleotide-directed mutagenesis by the procedure of Kunkel (15). The mutation in the _sigE_ allele resulted in changing the methionine (M) to an alanine (A) at position 124 (sigE124MA). The nucleotide sequence of the oligonucleotide used to make the _sigE_124MA allele was 5'-TCTTAAATACGCCAGGATTCA-3'. To ensure that the proper mutant _sigE_ allele was constructed, the DNA sequence of the mutated gene was determined by using the oligonucleotide 5'-TACGGAAATATAG-3', which is complementary to a region in _sigE_.

Construction of _B. subtilis_ strains containing _sigE_ alleles under control of the _Pspac_ promoter. _B. subtilis_ strains containing _sigE_ alleles under _Pspac_ promoter control were constructed in two stages, as described previously for the construction of _sigA_ alleles controlled by _Pspac_ (9, 11). First, the _sigE_ and mutant _sigE_ alleles were cloned downstream from the _Pspac_ promoter in pAG58bleo-1. Second, the _Pspac_–_sigE_ alleles were cloned into pTV21Δ2 so they could be easily transferred into the chromosome. The plasmids pJ8903 and pJsigE124MA, containing the wild-type and mutant _sigE_ alleles, respectively, were digested with _PstI_, generating 1.1-kb fragments, which were made blunt-ended with T4 DNA polymerase and cloned into the _SstI_ site of pIC20H (18), creating pICsigE and pICsigE124MA. The _sigE_ alleles were excised from the plasmids as _XbaI_ fragments and ligated into the _XbaI_ site of pAG58bleo-1, generating plasmids pSPIGMA124MA and pSPIGMA124MA, respectively. These plasmids have the wild-type and mutant _sigE_ alleles under the control of the _Pspac_ promoter and are selectable by resistance to bleomycin in _E. coli_ and phleomycin in _B. subtilis_. To ensure that the wild-type and mutant _sigE_ alleles were cloned in the correct orientation with respect to the _Pspac_ promoter, the plasmids were characterized by restriction endonuclease analysis and by nucleotide sequencing analysis.

The _PstI-NcoI_ fragment from pSPIGMA plasmids, which contains the wild-type and mutant _sigE_ allele under the control of the _Pspac_ promoter, was excised and ligated to the two 5-kb _PstI-NcoI_ fragments from pTV21Δ2. The ligated mixture was used to transform a _B. subtilis_ strain that contained pTV21Δ2 to phleomycin resistance at a temperature permissive for plasmid replication, as described previously (9, 11). These transformants were sensitive to chloramphenicol. In this transformation, the homology from pTV21Δ2 adjacent to the _Pspac-sigE_ region allowed recombination with the resident pTV21Δ2 plasmid, generating recombinant plasmids pTVSPIGMA124MA and pTVSPIGMA sigE124MA. The pTVSPIGMA plasmids were sequenced to ensure that the proper mutations were maintained. These plasmids were recombined into the chromosome of EU100, which contained a region homologous to the _Tn917_ sequences in the plasmids and a deletion of the _sigE_ chromosomal locus. EU100 had been derived from PY852 (chr::_Tn917_HU160_ (11), which contains a silent transposon _Tn917_ insertion, by transformation to chloramphenicol resistance with linearized pTV21Δ2, generating EU9000. The wild-type _sigE_ allele was replaced in EU9000 with the gene for erythromycin resistance by transformation with chromosomal DNA from strain EU8701 (10) and selection for growth on LB agar supplemented with 1 μg of erythromycin per ml. A single transformant (EU100) which was resistant to both erythromycin at 1 μg/ml and chloramphenicol at 5
μg/ml and formed less than 10 heat-resistant spores per ml was chosen. B. subtilis EU100 was transformed with pTVSPIGMasigE and pTVSPIGMasigE124MA at the non-permissive temperature for plasmid replication and selected for growth on phleomycin with sensitivity to chloramphenicol, creating EU101 and EU102, respectively.

**β-Galactosidase assays.** B. subtilis EU101 and EU102 were lysogenized with the SPβ derivatives that contained wild-type and mutant *spoIID* promoters fused to lacZ and were selected by growth on LB supplemented with 5 μg of chloramphenicol per ml. Only those transductants that were able to grow on DS medium containing 5 μg of chloramphenicol, 0.5 μg of phleomycin, and 1 μg of erythromycin per ml were used for further analysis. DS medium containing chloramphenicol (5 μg/ml) was inoculated with a fresh transductant and grown until an OD₆₀₀ of 0.6 was attained, at which time the cultures were divided. To one half of the cultures, 1 mM IPTG (isopropylthiogalactopyranoside) was added. At hourly intervals, 1 ml of the cell culture was harvested, and β-galactosidase assays were done as described previously (10).

**RESULTS**

**Primer extension analysis of the *spoIID* transcript.** We used primer extension analysis to map the 5′ end of the *spoIID* transcript. The promoter for *spoIID* was thought to be located less than 230 bp upstream from the *spoIID* coding region, since a DNA fragment containing this region and the coding region could complement *spoIID* mutations in *trans* when inserted in the *amy* locus of the B. subtilis chromosome (14). Two different primers were used in R. Losick’s laboratory to map the 5′ end of the *spoIID* transcript (13, 28). We confirmed their results with a third primer. Our primer extension analysis identified a transcript, which was weakly detected 2 h after the onset of sporulation but was abundant 4 h after the onset of sporulation (Fig. 1). Comparison of the electrophoretic mobility of the primer extension product with that of the products of the dideoxy sequencing reactions in Fig. 1 was used to map the 5′ end of the transcript on the DNA sequence. The primer extension product comigrated with a dideoxyguanosine product (Fig. 1) that corresponds to C in the nontranscribed strand (Fig. 2), 160 bases upstream from the start of the *spoIID* coding sequence (14). The 5′ end of the *spoIID* transcript probably represents the end of the primary transcription product rather than a 5′ end that was generated by processing of a longer transcript, since a similar transcript was generated in vitro by RNA polymerase containing σ⁸ (data not shown).

We assigned the start point of transcription to the adenine residue located 1 bp downstream from the cytosine (Fig. 2) because in in vitro transcription experiments with σ⁸ RNA polymerase, the dinucleotide ApA primed transcription from this promoter more efficiently than did ApC (data not shown). Direct evidence that the sequence upstream from this putative start point of the *spoIID* transcript acts as the promoter was provided by the mutagenesis experiments described below.

**Analysis of *spoIID* promoter mutations.** To determine whether the DNA surrounding the putative start of the *spoIID* transcript had promoter activity in vivo and to examine the effects of mutations in this promoter, we cloned a DNA fragment containing this region (from 72 bp upstream from the transcription start point to 63 bp downstream from the transcription start point) upstream from a promoterless derivative of lacZ from *E. coli* as described in Materials and

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**FIG. 1.** Primer extension analysis of *spoIID* transcription. Total RNA was isolated from *B. subtilis* at 0 (lane a), 2 (lane b), and 4 h (lane c) after the onset of sporulation. An oligonucleotide primer was hybridized to the RNA and extended with reverse transcriptase. The resulting cDNAs were subjected to electrophoresis on polyacrylamide sequencing gels next to size standards that were produced by using the same oligonucleotide in dideoxy sequencing reactions with a DNA template containing the *spoIID* promoter region (lanes d through g). The position of the primer extension product is indicated by the arrows.

Methods. This *spoIID-lacZ* fusion was carried into the chromosome of *B. subtilis* on a SPβ specialized transducing phage. We found that β-galactosidase began to accumulate about 2 h after the onset of sporulation in an otherwise wild-type strain that had been lysogenized with the *SPβIID-lacZ* transducing phage (data not shown).

To determine whether the region upstream from the start point of transcription was promoting this expression of lacZ, we introduced two different single-base-pair substitutions—a transition and a transversion, 13 and 12 bp, respectively, upstream from the putative start point of transcription (Fig. 2). These specific base substitutions were chosen based on our previous studies of seven promoters that are used in vitro by σ⁸ RNA polymerase (21). In these studies, we found

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**FIG. 2.** Nucleotide sequence of DNA near the start point of *spoIID* transcription. The sequence of the nontranscribed strand is shown. The start point of transcription is indicated as +1. The transition and transversion mutations located 13 and 12 bp, respectively, upstream from the transcription start point are indicated. Shown above the DNA sequence is the amino acid sequence, represented as an α-helix in the one-letter code, of the region of σ⁸ that may interact with the −10 region of the *spoIID* promoter.
FIG. 3. The −10 recognition region of several sigma factors. Shown are the amino acid sequences of the region in sigma factors that may interact with the −10 regions of promoters. The sigma factors are from B. subtilis except for two from E. coli, σ32 and σ32. The circled positions indicate the positions at which amino acid substitutions have been found to specifically suppress the effects of mutations in the −10 region of the spoIID promoter (AAATACG) (Fig. 2). In our previous studies of the effects of mutations in two promoters (21), we found that a single substitution of a G at the first position of the consensus created promoters that were used less efficiently by σE RNA polymerase. Therefore, we made this substitution at position −13 in the spoIID promoter. We also substituted T for A at the position in the spoIID promoter (−12) that may be homologous to the second position of the consensus sequence, since T was not found at this position in any of the promoters used by σE RNA polymerase in vitro (21). We also inserted mutant promoters into the −10 region of the spoIID promoter in vivo, these mutations would probably affect promoter activity if transcription were being initiated from this sequence, because the nucleotide sequences at the −10 region of most types of promoters are important determinants of promoter strength (19). Both of these single-base substitutions reduced the promoter activity of this region. These mutant derivatives of this promoter provided us with the opportunity to test whether an amino acid substitution in σE could suppress the effect of a mutation in this promoter.

Suppression of the effect of a promoter mutation by an amino acid substitution in σE. The positions at which amino acid substitutions in σ32, σA, and σD have been found to suppress the effects of single-base-pair substitutions in the −10 region of their cognate promoters lie within conserved regions of these sigma factors (Fig. 3). It is likely that most of these sigma factors use a similar motif to interact directly with the −10 regions of their cognate promoters. If σE interacts at the −10 region of its cognate promoters in a manner similar to that of σA, then possibly the side chain of the methionine at position 124 of σE, like its homolog in σA (the threonine at position 100), would interact near position −13 of its cognate promoters (Fig. 3). If this model is correct, then alteration of the side chain at position 124 by substitution of alanine for methionine may produce a form of σE that is unable to discriminate between two promoters that differ only by the base pair at position −13. This type of loss-of-specificity mutation has been observed with σA(1) as well as with sequence-specific DNA-binding proteins (3).

To test this model, we constructed strains that contained the structural gene for σA, sigE, or the mutant sigE allele was fused to the inducible promoter Ppsac. The Ppsac-sigE fusions, lacI (which encodes the repressor of Ppsac), and ble (which encodes resistance to phleomycin) were inserted into the chromosome of a sigE deletion strain of B. subtilis by homologous recombination with Trn917 as described in Materials and Methods. In order to examine the effects of the sigE alleles, each strain also carried a SPβ prophage that contained lacZ fused to the spoIID promoter or mutant derivatives of this promoter, which differed by a single-base-pair substitution.

DISCUSSION

We have defined the spoIID promoter in three steps. We first determined the location of the 5′ end of a transcript that is initiated upstream from the spoIID structural gene about 2 h after the onset of sporulation. We next showed that the DNA fragment containing this region exhibited promoter activity when fused to lacZ in B. subtilis and that single-
FIG. 5. Effects of wild-type and mutant sigE alleles on expression of spoIIID-lacZ expression. At time zero (about 20 min before the end of exponential growth), the cultures were split and IPTG was added to half the cultures. The allele of sigE fused to \( \text{P}_{\text{ spo }} \) is indicated above each panel. The positions of the base pair substitutions in the spoIIID promoter are also indicated above each panel. Symbols: \( \square \), expression in uninduced cultures; \( \blacksquare \), activity following induction. Each point represents the average value from two experiments.

base-pair substitutions at two positions in the -10 region of this promoter reduce its activity. In other work (7), we have shown that this promoter (\( \text{spoIIID} \)) is used efficiently in vitro by \( \sigma^E \) RNA polymerase.

We had previously noted conserved sequences at the -10 and -35 regions of promoters that are used by \( \sigma^E \) RNA polymerase in vitro (21). Recently, Foulger and Errington (4) have compiled additional sequences of promoters that are probably used by \( \sigma^E \) RNA polymerase. (They included the \( \text{spoIIID} \) promoter in their list of sequences.) They used these sequences to suggest a modified consensus for the -10 and -35 regions of promoters used by \( \sigma^E \) RNA polymerase. Their consensus for the -10 region of \( \sigma^E \)-dependent promoters retains the most highly conserved features of the sequence found previously (21) but did not include the C that was noted at the first position of the -10 region (4). This position is occupied by an A in the \( \text{spoIIID} \) promoter, and we found that substitution of a G at this position decreased promoter activity. Evidently, the base pair at this position plays an important role in signaling recognition of the promoter by \( \sigma^E \) RNA polymerase. In fact, the results of the suppression experiment suggest that this base pair interacts with the amino acid at position 124 of \( \sigma^E \). The addition of the new sequences of \( \sigma^E \)-dependent promoters by Foulger and Errington (4) and recently by R. Losick and his colleagues (16) probably increases the accuracy with which the consensus sequence represents the sequences that signal recognition of promoters by \( \sigma^E \) RNA polymerase. However, this assumption must be tested by additional mutagenesis of promoters that are used by \( \sigma^E \) RNA polymerase.

The correlation of \( \text{spoIIID} \) promoter activity with the production of \( \sigma^E \) during sporulation and its dependence on a functional allele of sigE had been used to suggest that \( \sigma^E \) directs expression from the \( \text{spoIIID} \) promoter (14, 25). These results, taken together with our results that show allelic-specific suppression of a \( \text{spoIIID} \) promoter mutation by an amino acid substitution in \( \sigma^E \), provide a compelling argument that the \( \text{spoIIID} \) promoter is used by \( \sigma^E \) RNA polymerase during sporulation.

During sporulation, \( \text{spoIIID} \) promoter activity is restricted predominantly to the mother cell compartment (14). Since the activities of several other promoters, thought to be used by \( \sigma^E \), are also restricted to the mother cell compartment, it has been suggested that \( \sigma^E \) activity is restricted to this compartment (2, 25). This model predicts that the activities of all promoters used by \( \sigma^E \) RNA polymerase are restricted to the mother cell compartment. In this regard, one of the important implications of our results is that the 124MA allele of sigE provides a tool with which to test whether specific promoters are used by \( \sigma^E \) RNA polymerase during sporulation. Specific suppression of a base substitution in a promoter by the 124MA allele of sigE would provide evidence that the promoter is used by \( \sigma^E \) RNA polymerase during sporulation.

Our results confirm and extend the observations that suggest that most sigma factors use a similar motif to make sequence-specific contacts with the -10 regions of their cognate promoters. Our results suggest that the methionine at position 124 of \( \sigma^E \) interacts with the base pair at position -13 of the \( \text{spoIIID} \) promoter, whereas \( \sigma^H \) uses a threonine for the homologous interaction with its cognate promoters (1, 32) (Fig. 3). Suppression experiments also support the model that the arginine at position 96 in \( \sigma^H \) contacts position -12 of the spoVG promoter (1) and that the glutamine at
position 196 in σA (and 437 in σ30 [29]) contacts position −12 in the lacBS promoter (9). Therefore, it seems likely that the asparagine at position 120 in σE has a homologous function (Fig. 3). The role of the other amino acids in this region of each sigma factor remains to be discovered. However, since σB is not essential for growth of B. subtilis and since it appears to interact with promoters in a manner similar to that of other sigma factors, examination of the effects of additional amino acid substitutions in σB can be expected to provide additional insights into the function of sigma factors.

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