Combined Action of Two Transcription Factors Regulates Genes Encoding Spore Coat Proteins of *Bacillus subtilis*

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During sporulation of *Bacillus subtilis*, spore coat proteins encoded by cot genes are expressed in the mother cell and deposited on the forespore. Transcription of the cotB, cotC, and cotX genes by αK RNA polymerase is activated by a small, DNA-binding protein called GerE. The promoter region of each of these genes has two GerE binding sites. 5′ deletions that eliminated the more upstream GerE site decreased expression of lacZ fused to cotB and cotX by approximately 80% and 60%, respectively but had no effect on cotC-lacZ expression. The cotC-lacZ fusion was expressed later during sporulation than the other two fusions. Primer extension analysis confirmed that cotB mRNA increases first during sporulation, followed by cotX and cotC mRNAs over a 2-h period. *In vitro* transcription experiments suggest that the differential pattern of cot gene expression results from the combined action of GerE and another transcription factor, SpoIID. A low concentration of GerE activated cotB transcription by αK RNA polymerase, whereas a higher concentration was needed to activate transcription of cotX or cotC. SpoIID at low concentration repressed cotC transcription, whereas a higher concentration only partially repressed cotX transcription and had little effect on cotB transcription. DNase I footprinting showed that SpoIID binds strongly to two sites in the cotC promoter region, binds weakly to one site in the cotX promoter, and does not bind specifically to cotB. We propose that late in sporulation the rising level of GerE and the falling level of SpoIID, together with the position and affinity of binding sites for these transcription factors in cot gene promoters, dictates the timing and level of spore coat protein synthesis, ensuring optimal assembly of the protein shell on the forespore surface.

Upon starvation, the Gram-positive bacterium *Bacillus subtilis* initiates a sporulation process involving a series of morphological changes (1). The rod-shaped cell undergoes asymmetrical division into two compartments, a larger mother cell and a smaller forespore. Different sets of genes are expressed from the genome in each compartment. As sporulation proceeds, the forespore is engulfed by the mother cell, forming a free protoplast surrounded by a double membrane inside the mother cell. Cell wall-like material called cortex is then deposited between the forespore membranes. Transcription of cot genes, which encode spore coat proteins, occurs in the mother cell. The coat proteins assemble on the surface of the forespore, forming a tough shell that protects the spore from environmental insults after it is released by lysis of the mother cell. When nutrients become available again, the spore germinates, producing a cell that resumes growth and division.

The sporulation process of *B. subtilis* has been studied as a model to understand the relationship between developmental morphogenesis and gene regulation (2). A central feature of sporulation gene regulation is the synthesis and activation of four compartment-specific σ subunits of RNA polymerase (RNAP).² σF and σG direct RNAP to transcribe genes in the forespore. σE and σF direct transcription in the mother cell. The four σ factors form a regulatory cascade in which the activation of each σ depends upon the activity of the prior σ in the order σF, σG, σE, and finally σK (3). Activation of each of the latter three σ factors appears to be coupled to a morphological step in development and involves signaling between the two compartments.

In the mother cell, two accessory transcription factors, SpoIIID and GerE, modulate RNAP activity at specific promoters (2). GerE is an 8.5-kDa protein that binds to DNA sequences resembling RWWTRGGY−YY (R is purine, W is A or T, and Y is pyrimidine) and activates transcription of many cot genes by αK (4, 5). GerE can also act as a repressor (6). Likewise, SpoIID is a 10.8-kDa protein that binds to DNA sequences resembling WWRACAR−Y and activates or represses transcription of many different genes (7, 8).

We have investigated transcriptional regulation of the cotB, cotC, and cotX genes. These genes were known to be transcribed by αK RNAP, with activation by GerE (4, 5). Two GerE binding sites had been mapped in the promoter region of each gene (Fig. 1) (4, 5). Here we report that 5′ deletions that eliminated the more upstream GerE site reduced expression of cotB and cotX but not cotC. Interestingly, we found that the three genes are differentially expressed during development, suggesting an additional level of regulation. SpoIIID appears to provide the additional control, based on the results of *in vitro* transcription and DNase I footprinting experiments presented here and based on how the SpoIIID level has been shown to change during sporulation (9, 10). This is the first study to correlate differential transcription of cot genes with the combined action of GerE and SpoIIID. The discovery of such complex regulation of cot gene expression leads us to speculate that synthesis of coat proteins is finely tuned to ensure optimal assembly of the spore coat.

### MATERIALS AND METHODS

**Construction of cot-lacZ Fusion Strains**—DNA fragments containing the cotB, cotC, or cotX promoter region flanked by EcoRI and HindIII restriction sites at the upstream and downstream ends, respectively,

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1 The abbreviations used are: RNAP, RNA polymerase; PCR, polymerase chain reaction; R, purine; W, A, or T; Y, pyrimidine.
were synthesized by the聚合ase chain reaction (PCR) and directionally subcloned into EcoRI-HindIII-digested pTkIac (11). The templates of the PCR were pBD136 (4), pHI1 (4), and pJZZ2 (5), respectively, for cotB, cotC, and cotX. Plasmids containing the cotB promoter region from −85 to +37 (pHI1) or from −60 to +37 (pHI4) were constructed using the upstream primer 5′-GGGAATTCGCGTGAAAATGG-GAT3′ or 5′-GGGAATTCAAGGACAATTTGCGT3′ for pH1 and pH4, respectively, and the downstream primer 5′-GGGAAGCTTAAT-TCCTCTAGTCA3′ (the restriction site in the primer is underlined). Plasmids containing the cotC promoter region from −153 to +15 (pHI6) or from −79 to +13 (pHI7) were constructed using the upstream primer 5′-GGGAATTCGCGTGAAAATGG-GAT3′ or 5′-GGGAATTCAAGGACAATTTGCGT3′ for pH1 and pH7, respectively, and the downstream primer 5′-GGGAAGCTTAAT-TCCTCTAGTCA3′.

Measurement of β-Galactosidase Activity—Sporulation was induced by nutrient exhaustion in Difco sporulation medium at 37°C as described previously (14). Samples (1 ml) were collected at hourly intervals during sporulation, cells were pelleted, and pellets were stored at −20°C before the assay. The specific activity of β-galactosidase was determined by the method of Miller (15), using o-nitrophenol-β-D-galactopyranoside as the substrate. One unit of enzyme hydrolyzes 1 μmol of substrate/min/unit of initial cell absorbance at 595 nm.

Primer Extension Analysis—At hourly intervals from 3 to 7 h after the onset of sporulation, cells were harvested by centrifugation (11,950 × g for 10 min), and RNA was prepared as described previously (16) except the RNA was resuspended in 100 μl of water that had been treated with 0.1% (v/v) diethylpyrocarbonate. The RNA was treated with DNase I to remove contaminating chromosomal DNA. Primer extension reactions were performed as described previously (17, 18). The cotB and cotC primers were those designated as Py2 previously (19). The cotX primer we used was also called Py2 previously (5). After the reaction, the extension products were subjected to electrophoresis in a 5% polyacrylamide gel containing 8 M urea, and transcripts were detected by autoradiography. The signal intensities were quantified using a Storm 820 PhosphorImager (Molecular Dynamics).

In Vitro Transcription—α32P RNA polymer was partially purified from gerE mutant cells as described previously (20). The enzyme was comparable in protein composition and in cotD- and sigK-transcribing activities with fraction 24 shown in Fig. 2 of Kroos et al. (20). GerE was gel-purified from Escherichia coli engineered to overproduce the protein as described previously (4). SpolIID was gel-purified from fractions of poly(dA-dT) or poly(dI-dC) as compared with cotC or cotX probe, respectively, was added as competitor. After DNase I treatment, the partially digested DNAs were electrophoresed in a 7% polyacrylamide gel containing one or the other of the PCR primers labeled at the 5′ end by treatment with T4 polynucleotide kinase and γ-32P ATP and purified by passage through a MicroSpin G-25 Column (Amersham Pharmacia Biotech). DNA probes for analysis of the cotX promoter region were prepared as described previously (5). Labeled DNA fragments were incubated with different amounts of gel-purified SpolIID and then mildly digested with DNase I according to method 2 of Zheng et al. (4), except 0.4 units of DNase I was used, and a 7-fold (w/w) excess of poly(dA-dT) or poly(dI-dC) as compared with cotC or cotX probe, respectively, was added as competitor. After DNase I treatment, the partially digested DNAs were electrophoresed in a 7% polyacrylamide gel containing 8 μM urea along side a sequencing ladder generated with T7 Sequenase V 2.0 (Amersham Pharmacia Biotech) and the appropriate primer for cotC or by chemical cleavage of the appropriate end-labeled DNA for cotX.

RESULTS

Role of Upstream GerE Binding Sites in cot Gene Transcription—The cotB, cotC, and cotX promoter regions each have two GerE binding sites (Fig. 1) (4, 5). To test the importance of the more upstream GerE site in transcription of each gene, we fused promoter DNA fragments containing different amounts of upstream sequence to lacZ. These fusions were recombined into the lysogenic phage SP8, and each phage was transduced into wild-type and gerE mutant B. subtilis, where the phase integrated into the chromosome at the attachment site. Transductants were induced to sporulate by nutrient exhaustion, and β-galactosidase activities were measured. Fig. 2 shows that deletion of the more upstream GerE site reduced cotB-lacZ and cotX-lacZ expression by approximately 80% and 60%, respectively. Deletion of the more upstream site centered at −134.5 had no effect on cotC-lacZ expression. All the fusions failed to be expressed in the gerE mutant (Fig. 2 and data not shown). These results demonstrate that the more upstream GerE site is not important for cotC transcription and suggest that the more upstream sites contribute greatly to GerE transcriptional activation of cotB and cotX.

cot Genes Exhibit Different Patterns of Expression—The data in Fig. 2 suggest that the cotB, cotX, and cotC promoters are regulated differently. Expression of cotB-lacZ rose sharply between 4 and 5 h into sporulation and reached a maximum at cotX-lacZ (5) and decreased after hours 4 and 5 but continued to rise until hour 7. Expression of cotC-lacZ began later, between hours 5 and 6, and rose until hour 7.

To further examine the apparent difference in the pattern of cot gene expression, we measured the appearance of cotB, cotX, and cotC mRNAs during sporulation of wild-type cells using primer extension analysis. Fig. 3A shows a representative re-
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FIG. 2.Expression of cot-lacZ fusion. cotB (A), cotX (B), and cotC (C) promoter regions with (□) or without (○) the more upstream GerE site (5’-end points are indicated in Fig. 1) were fused to lacZ, and β-galactosidase activity during sporulation of wild-type SG38 was measured as described under "Materials and Methods." Likewise, expression of fusions containing the more upstream GerE site was measured during sporulation of gerE mutant 522.2 (□). Points on the graph are averages for isolates of each type, and error bars show 1 S.D. of the data.

FIG. 3. Levels of cotB, cotC, and cotX mRNA during sporulation. RNA was prepared from wild-type SG38 collected at the indicated number of hours after the onset of sporulation in Difco sporulation medium. A, cotB, cotC, and cotX mRNA was detected by primer extension analysis. B, primer extension signals for cotB (□), cotC (○), and cotX (□) mRNA were quantified and normalized to the maximum signal for each mRNA. Points on the graph are averages of normalized signals from RNA prepared from two different cultures, and error bars show 1 S.D. of the data.

As a result of the experiment in which primers for all three mRNAs were mixed with RNA, and primer extension was done simultaneously. Similar results were obtained when primer extension was done separately for each gene (data not shown). cotB mRNA was detectable at 4 h into sporulation, and its level rose sharply at hour 5. Reproducibly, cotB mRNA was undetectable at hour 6, then reappeared at hour 7, indicating that synthesis and/or stability of this mRNA is regulated by an unknown mechanism late in development. cotC mRNA was barely detectable at hour 4, rose to its maximum level at hour 5, and fell to a barely detectable level at hour 6. cotC mRNA was present at a low level at hour 3. The enzyme responsible for this low level of cotC transcription is unknown, but it could be σK RNAP because σK and σE recognize similar sequences in cognate promoters (22, 23). The cotC mRNA level increased at hour 5 and continued to rise until hour 7. Fig. 3B shows quantification of the experiment shown in Fig. 3A plus one independent experiment. The average level at different times is plotted relative to the maximum level for each mRNA to illustrate the different patterns of mRNA accumulation. These results together with the lacZ expression data (Fig. 2) suggest that cotB transcription is induced slightly earlier than that of cotX, whereas full induction of cotC transcription lags behind that of cotX.

A Lower Concentration of GerE Activates cotB Transcription than cotX or cotC Transcription—To investigate how different patterns of cot gene expression might be established, we performed in vitro transcription with σK RNAP and different amounts of GerE. Fig. 4 shows a representative experiment in which an equimolar mixture of cotB, cotX, and cotC DNA templates was transcribed by σK RNAP (partially purified from

FIG. 4. Effect of GerE on cotB, cotC, and cotX transcription in vitro. A, DNA templates (0.05 pmol of each template) were transcribed with partially purified σK RNAP (0.2 μg) alone (lane 1) or with 6 (lane 2), 13 (lane 3), 25 (lane 4), 50 (lane 5), 100 (lane 6), 200 (lane 7), or 400 pmol (lane 8) of gel-purified GerE added immediately after the σK RNAP. DNA templates were a 478-base pair PvuII fragment of pBD136 (170-base cotB transcript), a 338-base pair EcolIII-CotH fragment of pH11 (196-base cotC transcript), and a 179-base pair PCR-generated fragment (82-base cotX transcript). The positions of run-off transcripts of the expected sizes, as judged from the migration of end-labeled DNA fragments of MspI-digested pBR322, are indicated. B, transcript signals for cotB (□), cotC (○), and cotX (□) were quantified and normalized to the maximum signal for each transcript. Points on the graph are averages of normalized signals from two experiments, and error bars show 1 S.D. of the data.
gerE mutant cells) in the presence of increasing GerE. In this in vitro system, all three genes were transcribed in the absence of GerE, whereas expression of lacZ fused to these genes was not observed in gerE mutant cells (Fig. 2). The addition of GerE activated transcription of all three genes in vitro, as expected (4, 5), but interestingly, a lower concentration of GerE was sufficient to activate cotB transcription, whereas a higher concentration was needed to activate cotX or cotC transcription (Fig. 4A). The experiment was repeated, and transcript signals from both experiments were quantified and normalized to the maximum signal obtained for each template, Fig. 4B shows that, on average, cotB transcription was activated about 3-fold and 0.5 μM GerE was required for half-maximal activation. The activation profiles for cotX and cotC were very similar. Both genes were activated more than 10-fold, and half-maximal activation required 4 μM GerE. These results suggest that earlier expression of cotB during sporulation (Figs. 2 and 3) may result from a lower threshold for activation by GerE, since the level of GerE is believed to increase as αK RNA polymerase becomes active (24). The results do not explain the apparent differential expression of cotX and cotC (Figs. 2 and 3), suggesting there might be an additional level of control.

**SpoIID is a Potent Repressor of cotC Transcription**—We discovered that extracts of sporulating wild-type cells contain a protein that binds to the cotC promoter region (data not shown). The kinetics of appearance of this binding activity and its absence from extracts of spoIID mutant cells suggested that the protein is SpoIID. The SpoIID protein had been shown previously to inhibit transcription of the cotD gene in vitro and to bind in the −35 region of the cotD promoter (7, 20). We hypothesized that SpoIID might contribute to the differential regulation of cot gene expression we had observed (Figs. 2 and 3). This hypothesis is difficult to test in vivo, because SpoIID is required for production of αK RNA polymerase (7, 25), which transcribes the cot genes (4, 5). To test whether SpoIID affects cot gene transcription in vitro, we modified the experiment shown in Fig. 4. Different amounts of SpoIID were incubated with a mixture of DNA templates before the addition of αK RNA polymerase and a fixed amount of GerE. In this set of experiments, equimolar GerE template was included as a control because we knew that SpoIID has very little effect on its transcription (26). Fig. 5A shows a representative result, and Fig. 5B summarizes quantification of two experiments. SpoIID repressed cotC transcription about 10-fold, with 50% repression occurring at 0.2 μM. cotX transcription was repressed about 2-fold at the highest SpoIID concentration tested (approximately 1 μM). SpoIID had very little effect on transcription of cotB or gerE. These results provide a plausible explanation for the lag between cotX and cotC expression (Figs. 2 and 3). The level of SpoIID decreases as active αK RNA polymerase accumulates in the mother cell (9, 10). Our in vitro transcription results suggest that cotX would be released from SpoIID repression first, followed by cotC when the SpoIID concentration reaches a much lower level.

**SpoIID Binds to Specific Sites in the cotC and cotX Promoter Regions**—The inhibitory effect of SpoIID on cotC and cotX transcription suggested that SpoIID might bind to specific DNA sequences in the promoter regions of these genes. To examine specific binding by SpoIID, we performed DNase I footprinting experiments. SpoIID protected two regions of cotC promoter DNA from digestion with DNase I. The protection spanned positions −43 to −29 and positions −77 to −56 on the transcribed strand (Fig. 6A). On the nontranscribed strand, protection spanned positions −40 to −22 and positions −75 to −63 (Fig. 6B). Protection was observed at the lowest concentration of SpoIID tested, indicating that SpoIID binds with relatively high affinity to these sites as compared with other SpoIID binding sites mapped previously (7, 8). Fig. 6C shows the sequence of the cotC promoter in the two regions protected by SpoIID. Within each protected region is a sequence that matches the consensus sequence for SpoIID binding (Fig. 6D). These results may explain why SpoIID is a potent repressor of cotC transcription (Fig. 5). The upstream SpoIID binding site centered at position −67.5 (Fig. 6C) overlaps the critical GerE site centered at position −68.5 (Fig. 1). The downstream SpoIID binding site centered at −36.5 overlaps the −35 region of the cotC promoter, which may be important for recognition by αK RNA polymerase.

SpoIID also bound specifically to a site in the cotX promoter. Protection from DNase I digestion spanned positions −27 to −11 on the transcribed strand (Fig. 7A) and at least positions −23 to −15 on the nontranscribed strand (Fig. 7B). Fig. 7C shows the sequence of the cotX promoter in the region protected by SpoIID. Within this region is a sequence that matches the consensus sequence for SpoIID binding in 7 of 9 positions (Fig. 7D). SpoIID binds with relatively low affinity to this site in the cotX promoter (Fig. 7, A and B) as compared with the two sites in the cotC promoter (Fig. 6, A and B), which may explain why SpoIID was a less potent repressor of cotX transcription than cotC transcription (Fig. 5).
**FIG. 6.** SpoIIID footprints in the cotC promoter region. Radioactive DNA fragments separately end-labeled on the transcribed (A) or nontranscribed (B) strand were incubated in separate reactions with a carrier protein (bovine serum albumin, 310 pmol) only (lane 1) or with 4 (lane 2), 8 (lane 3), 15 (lane 4), 30 (lane 5), or 60 pmol (lane 6) of gel-purified SpoIIID in addition to the carrier protein and then subjected to DNase I footprinting in a total volume of 45 μl. Filled boxes indicate the region protected from DNase I digestion by SpoIIID. Arrowheads denote the boundaries of protection, and numbers to the left refer to positions relative to the transcriptional start site, as deduced from sequencing ladders generated with T7 Sequenase V 2.0 (Amer sham Pharmacia Biotech) and the appropriate primer. Asterisks indicate the position of sites rendered hypersensitive to DNase I digestion by SpoIIID binding. C, positions of SpoIIID footprints in the cotC promoter region. The nucleotide sequence of the nontranscribed strand of the cotC promoter region is shown (4). Nucleotides in the −35 region that match the consensus for recognition by ρ-dependent RNA polymerase (m indicates A or C) are shown as boldface capital letters. Overlining and underlining indicate regions on the nontranscribed and transcribed strands, respectively, protected by SpoIIID from DNase I digestion. The dashed lines indicate regions of uncertain protection due to a lack of DNase I digestion in these regions. Numbers refer to positions relative to the transcriptional start site. D, nucleotide sequences within the SpoIIID-protected region of the cotC promoter are aligned with the consensus sequence for SpoIIID binding. Matches to the consensus sequence are shown as capital letters, and numbers refer to positions relative to the transcriptional start site.

**FIG. 7.** SpoIIID footprints in the cotX promoter region. Radioactive DNA fragments separately end-labeled on the transcribed (A) or nontranscribed (B) strand were incubated in separate reactions with a carrier protein (bovine serum albumin, 310 pmol) only (lane 1) or with 5 pmol (lane 2), 10 pmol (lane 3), 20 pmol (lane 4), 40 pmol (lane 5), or 60 pmol (lane 6) of gel-purified SpoIIID in addition to the carrier protein and then subjected to DNase I footprinting in a total volume of 45 μl. See the Fig. 6 legend for explanation of filled boxes, arrowheads, numbers to the left, and asterisks. C, position of the SpoIIID footprint in the cotX promoter region. The nucleotide sequence of the nontranscribed strand of the cotX promoter region is shown (39). Overlining and underlining indicate regions on the nontranscribed and transcribed strands, respectively, protected by SpoIIID from DNase I digestion. The dashed lines indicate regions of uncertain protection due to a lack of DNase I digestion in these regions. Numbers refer to positions relative to the transcriptional start site. D, nucleotide sequences within the SpoIIID-protected region of the cotX promoter are aligned with the consensus sequence for SpoIIID binding. Matches to the consensus sequence are shown as capital letters, and numbers refer to positions relative to the transcriptional start site.

**DISCUSSION**

Our results strongly support the idea that the combined action of GerE and SpoIIID produces differential patterns of cot gene expression during *B. subtilis* sporulation. Previously, cotB and cotC had been thought to be coordinately regulated by the appearance of GerE (4, 19). However, expression of a cotB-lacZ fusion begins to increase at least 1 h earlier than expression of a cotC-lacZ fusion (Fig. 2), and cotB mRNA reaches its maximum level at least 2 h earlier than cotC mRNA (Fig. 3). The earlier expression of cotB during sporulation may result, in part, from a lower threshold for activation by GerE (Fig. 4), but in addition, SpoIIID was shown to be a potent repressor of cotC transcription (Fig. 5). The repressive effect of SpoIIID on cotC transcription in vitro appears to be due to the presence of two relatively high affinity SpoIIID binding sites in the cotC promoter region that overlap binding sites for GerE and ρ-dependence RNA polymerase (Fig. 6). Therefore, we propose that SpoIIID represses cotC transcription during sporulation, contributing to the observed lag between cotB and cotC expression.

Consideration of our results with the cotX promoter provides additional support for the proposal that SpoIIID delays full expression of cotC during sporulation. The pattern of cotX-lacZ expression and cotX mRNA accumulation was more similar to that of cotB than cotC (Figs. 2 and 3), yet cotX and cotC transcription in vitro exhibited similar dependence on the concentration of GerE (Fig. 4), providing no explanation for the observed differential expression of cotX and cotC in vivo. This difference can be plausibly explained by our finding that SpoIIID is a more potent repressor of cotC transcription in vitro than of cotB (Fig. 5). SpoIIID appears to be a weak repressor of cotX because it binds with relatively low affinity to a site in the promoter that overlaps the binding site for ρ-dependence RNA polymerase (Fig. 7). If SpoIIID does repress transcription from the cotX promoter during sporulation, this repression would be expected to be relieved earlier than repression of cotC, as the level of SpoIIID decreases in the mother cell (9, 10).

Differential timing of cotB and cotC expression was overlooked previously due to hybridization of a primer that was thought to be cotC-specific with cgeAB mRNA (4, 19, 23). Hence, the primer extension analysis reported previously shows that cotB and cgeAB transcripts appear with similar timing during sporulation (19) and does not conflict with our finding that cotC expression lags behind that of cotB (Figs. 2 and 3). Expression of a cotC-lacZ fusion was shown previously to be induced about 1 h later during sporulation than expression of cotD-lacZ (27). Interestingly, the difference in time of
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Fig. 8. A model showing how the combined action of SpoIIID and GerE may regulate cot genes in the context of interactions between mother cell-specific transcription factors. Dashed arrows show gene (italicized)-to-product (proteins are circled) relationships. Solid arrows represent positive regulation of transcription. Lines with a barred end represent negative regulation of transcription. The boxed bars distinguish cotD and cotX, which are proposed to be weakly repressed by SpoIIID, from cotB and cgeAB, which are not repressed by SpoIIID, and from cotC, which is expressed later because it is strongly repressed by SpoIIID (indicated by the thick line with a barred end).

induction disappeared when the genes were artificially induced by production of \( \sigma^{K} \) during growth (27). Under these conditions, SpoIIID would not be present. Therefore, we propose that SpoIIID is responsible for the observed delay during sporulation in cotC expression as compared with that of cotD. A prediction of this hypothesis is that SpoIIID is a more potent repressor of cotC transcription than of cotD transcription. SpoIIID was shown previously to bind with relatively high affinity to a site spanning the −35 region of the cotD promoter and repress transcription in vitro (7); however, the effect of SpoIIID on cotD and cotC transcription in vitro has not been compared directly.

Fig. 8 illustrates how the combined action of SpoIIID and GerE may produce differential regulation of cot gene transcription in the context of known regulatory interactions with the two mother cell-specific factor \( \sigma^{K} \), \( \sigma^{D} \), and \( \sigma^{E} \). RNAP transcribes the spoIIID gene (28–32). As SpoIIID accumulates, it activates transcription of the sigK gene (7, 25). The primary product of the sigK gene, pro-\( \sigma^{K} \) (not shown in Fig. 8), is processed to \( \sigma^{K} \) in an activation step coupled to a signal from the forespore (33–35). \( \sigma^{K} \) RNAP transcribes gerE (4, 24). As GerE and \( \sigma^{K} \) RNAP accumulate, cotB (4, 19), cgeAB (23), and other genes begin to be transcribed. The other genes include cotD (4, 6, 7, 20) and cotX (5, 36), but we propose that SpoIIID limits transcription of these genes (boxed in Fig. 8) and prevents transcription of cotC for about 1 h. Repression by SpoIIID is relieved as its level declines due to degradation of the protein and due to a negative feedback loop initiated by \( \sigma^{K} \) RNAP that inhibits transcription of sigE and other early sporulation genes, thus inhibiting further production of \( \sigma^{K} \) and SpoIIID (9, 10, 37). The falling levels of \( \sigma^{K} \) and SpoIIID and the rising levels of \( \sigma^{D} \) and GerE together with the fact that both SpoIIID and GerE can act as activators or repressors of transcription (4, 6–8) make it possible to regulate the timing and level of individual cot gene transcription in a variety of ways.

Our 5′ deletion analysis of cot promoters gives further insight into the function of GerE as an activator of transcription. Deletions designed to eliminate the more upstream GerE binding site in the cotB and cotX promoters greatly reduced expression of lacZ fusions (Fig. 2), strongly suggesting that GerE binds to sites centered at −73.5 and −46.5 in the cotB and cotX promoters, respectively, and contributes to transcriptional activation of these promoters during sporulation. On the other hand, the finding that elimination of the more upstream GerE sites in these promoters did not abolish GerE-dependent expression suggests that the more downstream GerE sites are sufficient for weak transcriptional activation. The more downstream GerE site in the cotB promoter has the sequence 5′-AATTAGGCTATT-3′ (4), which matches perfectly the consensus sequence for GerE binding (5). This sequence is centered at −47.5 (4), which seems to be a preferred position for binding in promoters activated by GerE, since the cotVWX, cotYZ, and cotD promoters also have a sequence matching the consensus centered at −47.5 or −46.5, to which GerE appears to bind, activating transcription (5, 6). The more downstream site in the cotX promoter has the sequence 5′-GACTGAGTCATA-3′, which matches in 7 of 10 positions in the consensus sequence for GerE binding (5). This sequence is centered at −37.5 and is in the opposite orientation relative to the direction of cotX transcription as compared with the site centered at −47.5 in the cotB promoter. Assuming that GerE binds in a particular orientation to sequences similar to its nonpalindromic consensus sequence, our results suggest that GerE can activate transcription when bound in opposite orientations to sites centered at −47.5 and −37.5 (Figs. 1 and 2). Our results also suggest that GerE can activate transcription when bound to a site centered as far upstream as −73.5 in the cotB promoter or one-half turn of the DNA helix downstream at −68.5 in the cotC promoter. Hence, GerE may be less stringent than, for example, the E. coli catabolite gene activator protein with respect to the position from which it can activate transcription (38). This idea can be tested further by creating single base pair changes that eliminate GerE binding to individual sites and/or systematically varying the position of a GerE binding site in a promoter region.

The 5′ deletion we created that eliminates the more upstream GerE binding site in the cotX promoter may prove to be useful for investigating the mechanism of GerE transcriptional activation at this promoter. A recent study suggests that GerE may interact with \( \sigma^{K} \) at the cotX promoter and facilitate the initial binding of \( \sigma^{K} \) RNAP to the promoter (36). Certain amino acid substitutions in \( \sigma^{K} \) reduced expression of a cotX-lacZ fusion but not expression of a gerE-lacZ fusion, which also depends on \( \sigma^{K} \) RNAP but not on GerE. The authors speculated that GerE bound to the more downstream site centered at −37.5 contacts \( \sigma^{K} \), enhancing binding of \( \sigma^{K} \) RNAP to the cotX promoter. To explain the observation that the substitutions in \( \sigma^{K} \) did not eliminate cotX-lacZ expression, the authors proposed that GerE bound to the more upstream site centered at −60.5 makes a different contact with \( \sigma^{K} \) RNAP (e.g. with the C-terminal domain of the \( \sigma^{K} \) subunit). This model predicts that expression of the cotX-lacZ fusion we made, lacking the GerE site centered at −60.5, would be abolished in the mutants with amino acid substitutions in the \( \sigma^{K} \) region thought to interact with GerE.

Our results shed more light on how SpoIIID can function as a transcriptional repressor. SpoIIID footprints in the bofA, cotD, and spoVD promoter regions have been published previously (7, 8). In each case, SpoIIID binds to sites centered at +1 and/or −35, presumably preventing RNAP from binding to the promoter or hindering a subsequent step in transcription initiation. In the cotC promoter region, SpoIIID binds to a site centered at −67.5 (Fig. 6), which presumably prevents GerE from binding to its site centered at −68.5 (4), and SpoIIID binds to a site centered at −36.5 (Fig. 6), which presumably interferes with RNAP binding or a subsequent step in initiation. Likewise, SpoIIID binding to a site centered at −16.5 in the cotX promoter (Fig. 7) probably interferes with RNAP function.

Why are certain cot genes subject to dual regulation by SpoIIID and GerE? One possibility is that fine-tuning of cot gene expression allows optimal levels of Cot proteins to be
synthesized at the proper times for assembly into the spore coat. Our results suggest that expression of cotC is delayed relative to expression of other cot genes. We plan to test whether the delay in cotC expression is important by engineering cells to produce CotC earlier and measuring spore resistance properties. Fine-tuning of cot gene expression may also allow the spore coat to be suitably tailored in response to environmental conditions. Expression of a cotC-lacZ translational fusion was shown to depend strongly on whether sporulation was induced by sudden or gradual nutritional shift-down (19). Whether this regulation involves one of the previously known cotC transcription factors (i.e. GerE or σK RNAP), the newly discovered cotC repressor reported here (i.e. SpoIID), or some other mechanism remains to be elucidated.

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