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A Feedback Loop Regulates the Switch from One Sigma Factor to the Next in the Cascade Controlling *Bacillus subtilis* Mother Cell Gene Expression

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Regulation of gene expression in the mother cell compartment of sporulating *Bacillus subtilis* involves sequential activation and inactivation of several transcription factors. Among them are two sigma factors, σ^E and σ^K, and a DNA-binding protein, SpoIIID. A decrease in the level of SpoIIID is thought to relieve its repressive effect on transcription by σ^K RNA polymerase of certain spore coat genes. Previous studies showed that σ^K negatively regulates the level of *spoIIID* mRNA. Here, it is shown that σ^K does not affect the stability of *spoIIID* mRNA. Rather, σ^K appears to negatively regulate the synthesis of *spoIIID* mRNA by accelerating the disappearance of σ^E RNA polymerase, which transcribes *spoIIID*. As σ^K begins to accumulate by 4 h into sporulation, the σ^E level drops rapidly in wild-type cells but remains twofold to fivefold higher in *sigK* mutant cells during the subsequent 4 h. In a strain engineered to produce σ^K 1 h earlier than normal, twofold less σ^E than that in wild-type cells accumulates. σ^K did not detectably alter the stability of σ^E in pulse-chase experiments. However, β-galactosidase expression from a *sigE-lacZ* transcriptional fusion showed a pattern similar to the level of σ^E protein in *sigK* mutant cells and cells prematurely expressing σ^K. These results suggest that the appearance of σ^K initiates a negative feedback loop controlling not only transcription of *spoIIID*, but the entire σ^E regulon, by directly or indirectly inhibiting the transcription of *sigE*.

Sporulation of the gram-positive bacterium *Bacillus subtilis* is a model system for studying developmental gene regulation (8). In response to starvation, *B. subtilis* undergoes a series of morphological changes that culminate in the formation of an endospore. Early during sporulation, an asymmetrically positioned septum partitions the developing cell into two unequal compartments, the mother cell and the forespore, each of which carries a copy of the chromosome. The two compartments follow different programs of gene expression that drive further morphological changes, including migration of the septum to engulf the forespore, deposition of cell wall-like material called cortex between the two membranes surrounding the forespore, formation of a tough protein coat that encases the forespore, and lysis of the mother cell to release the endospore. Temporal and spatial gene regulation during sporulation is established by compartment-specific activation of a cascade of sigma factors, namely, σ^F, σ^E, σ^G, and σ^K, in order of their appearance (26, 34). The forespore-specific program of gene expression is controlled by σ^F and σ^G, while the mother cell program is controlled by σ^E and σ^K. Each sigma factor is initially inactive. σ^F is the first to become active, and this occurs only in the forespore (13, 32, 39). Activation of subsequent sigma factors in the cascade is triggered by signal transduction between the two compartments (12, 34). The inactive forms of the mother cell-specific sigma factors are precursor proteins called pro-σ^E and pro-σ^K. Each is synthesized about 1 h before it is activated by proteolysis (6, 30, 35).

Temporal gene regulation in the mother cell is established primarily by the ordered appearance of σ^E and then σ^K. Also involved is a transcription factor, SpoIIID, whose mRNA is synthesized by σ^E RNA polymerase (28, 49, 52). SpoIIID is a sequence-specific DNA-binding protein that activates or represses many different genes transcribed by σ^E and/or σ^K RNA polymerase (10, 27, 55). One of the genes activated by SpoIIID is *sigK*, which encodes pro-σ^K. The *sigK* gene is constructed during sporulation by a DNA rearrangement that joins *spoIVCB* (encoding the N-terminal part) and *spoIIIC* (encoding the C-terminal part) (51), and SpoIIID also activates transcription of *spoIVCA* (10, 45), the site-specific recombinase that catalyzes the rearrangement (29, 43, 46). Hence, SpoIIID plays a key role in progression from the early σ^E-directed pattern of gene expression to the late σ^K-directed pattern. Somewhat paradoxically, SpoIIID represses certain late genes in the σ^K regulon, apparently fine-tuning their timing and/or level of expression (10, 27, 56). How is the repressive effect of SpoIIID on late gene expression relieved? We showed previously that the SpoIIID protein level decreases abruptly when σ^K appears during sporulation (9). Also, in mutants that fail to make active σ^K, both SpoIIID and its mRNA persist at a higher level until later during sporulation compared to wild-type cells. This suggests that σ^K negatively regulates the synthesis and/or stability of *spoIIID* mRNA. As the existing SpoIIID is degraded, the σ^K-dependent genes that were repressed by SpoIIID would begin to be transcribed.

Here, we describe our continued investigation of the negative feedback loop connecting the production of σ^K to the SpoIIID decrease during sporulation. We demonstrate that σ^K does not affect the stability of *spoIIID* mRNA; therefore, it must exert its negative effect on *spoIIID* transcription. Indeed, a *spoIIID-lacZ* fusion is overexpressed in *sigK* mutant cells (28). Transcription of *spoIIID* is carried out by σ^E RNA polymerase (28, 49, 52). We show here that σ^K also negatively regulates the σ^E level, providing a simple explanation for the negative effect of σ^K on *spoIIID* transcription. σ^K directly or indirectly inhibits the transcription of *sigE* (encoding σ^E), based on the levels of expression from a *sigE-lacZ* transcriptional fusion in wild-type and different mutant strains. Thus,

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σ^K initiates a negative feedback loop that controls not only *spoIIID* expression but expression of the entire σ^E regulon.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* BK556 (*spoIVCB23*) (28), VO48 (*spoIVCBΔ19 cat*) (6), and SC776 (*bofB8 cat*) (6), which are isogenic with the wild-type Spo$^+$ strain PY79 (54), were provided by R. Losick. Strain BZ536 (P_{spac}-P_{sigK}-*sigKΔ19 spc*) was constructed by first replacing the *cat* allele of VO536 (P_{spac}-P_{sigK}-*sigKΔ19 cat*) (40) with a spectinomycin (*spc*) allele by using plasmid pCm::Sp (48) and then by using the chromosomal DNA of the resulting strain to transform competent PY79 cells and to select for a spectinomycin-resistant transformant.

General methods. Preparation of competent cells for transformation with plasmid DNA or chromosomal DNA was described previously (14). Sporulation was induced by resuspending growing cells in SM medium as described previously (14). The onset of sporulation (T_0) was defined as the time of resuspension. Use of the specialized transducing phage SPβ::*sigE-lacZ* (also called *spoIIG-lacZ*) has been described elsewhere (24). β-Galactosidase activity was assessed qualitatively by placing cells on DSM agar (14) containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (20 μg/ml) and was determined quantitatively with toluene to permeabilize cells and *o*-nitrophenol-β-D-galactopyranoside as the substrate (14). One unit of enzyme hydrolyzes 1 μmol of substrate per min per A_{600} of initial cell density.

Measurement of the stability of *spoIIID* mRNA. At the fourth hour of sporulation, rifampin (75 μg/ml) was added to cultures to stop transcription initiation. Samples (9 ml) were taken before and immediately after the addition of rifampin and were centrifuged at 12,000 × *g* for 1.5 min. Cell pellets were frozen in a dry ice-ethanol bath. Samples were also taken at 5 and 12 min after the addition of rifampin. The process of sample collection took about 3 min to complete; therefore, the first time point immediately after rifampin treatment was designated as 3 min after the stoppage of new transcription in cells. Likewise, the ensuing time points were designated as 8 and 15 min after the stoppage of transcription. RNA was prepared by the acid guanidinium thiocyanate-phenol-chloroform method (3) with the following modifications. Cell pellets were resuspended in 1.5 ml of denaturing buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) and 1.5 ml of acid phenol-chloroform (5:1) (Ambion) and then mixed vigorously with 1-ml glass beads (500 μm, acid washed; Sigma) to break cells. The mixture was centrifuged at 10,000 × *g* for 20 min at 4°C. After centrifugation, the aqueous phase was re-extracted with acid phenol-chloroform. RNA was precipitated by ethanol. Residual DNA was removed by digesting with RNase-free DNase. RNA (20 μg) was fractionated on a 1.2% (wt/vol) agarose gel containing 1.11% (vol/vol) formaldehyde, transferred to a nylon membrane, and hybridized to a random primed 1.1-kb DNA fragment containing the *spoIIID* coding sequence purified from pBK39 (28) digested with *Pst*I. The radioactive signals were quantified with a PhosphorImager (Molecular Dynamics).

Western blot analysis. Preparation of whole-cell proteins, electrophoresis, and electroblotting were described previously (9, 35). The membrane was probed with monoclonal anti-σ^E antibody (30) diluted 1:600 or polyclonal anti-SpoIIID antiserum (9) diluted 1:10,000. Chemiluminescence detection was performed according to the manufacturer's instructions (ECL; Amersham). When necessary, the membrane was then stripped of the bound antibodies and reprobed with polyclonal anti-pro-σ^K antiserum (35) diluted 1:10,000. Signals were quantified with a computing densitometer (Molecular Dynamics). Exposure times that gave maximum signal intensities within the linear response range of the X-ray film, as determined by control experiments, were used.

Pulse-chase and immunoprecipitation. At the third hour after the onset of sporulation, cells were pulse-labeled by adding [^{35}S]methionine (ICN) (35 μCi/ml) to the culture and incubating for 5 min. Excess (1,000-fold) unlabeled methionine and cysteine were then added, and the incubation was continued at 37°C. Cells (1 ml) were collected by centrifugation immediately following the pulse-chase and at 30-min intervals thereafter until the fifth hour after the onset of sporulation. The cells were frozen in a dry ice-ethanol bath and stored at −70°C.

Cell pellets were resuspended in 50 μl of lysis buffer (10 mM Tris-Cl [pH 8.4], 1 mM EDTA, 10 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.5-mg/ml lysozyme, 0.1-mg/ml DNase I) and incubated for 10 min at 37°C. Sodium dodecyl sulfate (SDS) was added to a concentration of 1%, and samples were boiled for 3 min. Lysates were centrifuged at 12,000 × *g* for 10 min. Lysates of different samples contained approximately equal amounts of radioactivity as judged by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography. After centrifugation, the supernatant was diluted 10-fold in immunoprecipitation buffer (50 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.2% deoxycholate, 1 mM phenylmethylsulfonyl fluoride). Monoclonal anti-σ^E antibody (80 μl), which was sufficient to quantitatively precipitate pro-σ^E and σ^E from a 1-ml culture in a control experiment, was added, and the mixture was incubated at 0°C for 2 h. A slurry (20 μl) of 1:1 (vol/vol) protein A-Sepharose CL-4B (Pharmacia)–immunoprecipitation buffer was then added, and the incubation was continued with gentle mixing on a rotary shaker at 4°C for 1.5 h. Samples were centrifuged briefly. The pellets were washed three times with 1 ml of immunoprecipitation buffer supplemented with 0.1% SDS and then resuspended in 30 μl of SDS sample buffer (31), boiled for 5 min, and centrifuged again to remove Sepharose beads. Immunoprecipitates were analyzed by SDS-PAGE. Pro-σ^E and σ^E bands were visualized by fluorography with ENTENSIFY (Dupont) as enhancing fluors and quantified by a PhosphorImager (Molecular Dynamics), with the background of each lane subtracted from the band intensity.

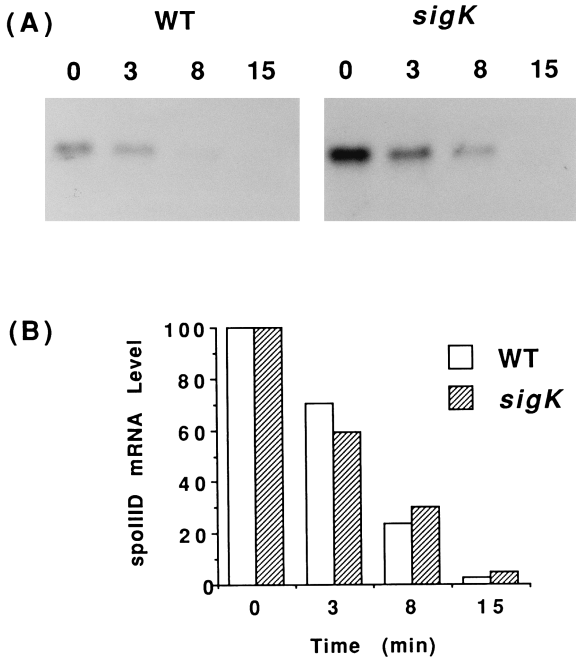

FIG. 1. The production of σ^K does not change the stability of *spoIIID* mRNA. (A) Wild-type (PY79) and *sigK* mutant (BK556) strains were induced to sporulate by resuspension in SM medium. Rifampin (75 μg/ml) was added to the medium at the fourth hour after the onset of sporulation. RNA was prepared from cells collected before and at the indicated number of minutes after the addition of rifampin, and equal amounts (20 μg) were analyzed by Northern blot analysis. (B) The level of *spoIIID* mRNA, as quantified by a PhosphorImager, is plotted as a percentage relative to the level before rifampin treatment. WT, wild type.

RESULTS

Stability of *spoIIID* mRNA in wild-type and *sigK* mutant cells. We showed previously that the *spoIIID* mRNA level reaches a higher maximum and remains higher late during sporulation of *sigK* (*spoIIIC94*; *spoIIIC* encodes the C-terminal part of σ^K) (51) mutant cells compared to that of wild-type cells (9). Similar results were obtained when cells containing another *sigK* mutation, *spoIVCB23* (*spoIVCB* encodes the N-terminal part of σ^K) (51) (both *spoIIIC94* and *spoIVCB23* cells fail to make σ^K) (35), were analyzed (data not shown). This *sigK* mutant was used in the studies reported here.

The higher level of *spoIIID* mRNA in the *sigK* mutants must be due to increased synthesis and/or stability of *spoIIID* mRNA. To measure the stability of *spoIIID* mRNA, sporulating wild-type and *sigK* mutant cells were treated with rifampin at T_4 (i.e., 4 h after starvation initiated sporulation) to stop transcription initiation. Total cellular RNA was isolated from cells collected before and at different times after the rifampin treatment. Northern blot analysis was performed to detect *spoIIID* mRNA. At T_4, there was already more *spoIIID* mRNA in *sigK* mutant cells than in wild-type cells (Fig. 1A), and a considerable amount of σ^K was present in the wild-type cells (data not shown). The amount of *spoIIID* mRNA remaining at different times after the rifampin treatment is shown in Fig. 1A and was quantified with a PhosphorImager. The half-life of *spoIIID* mRNA at T_4 is about 3.5 min in both wild-type and

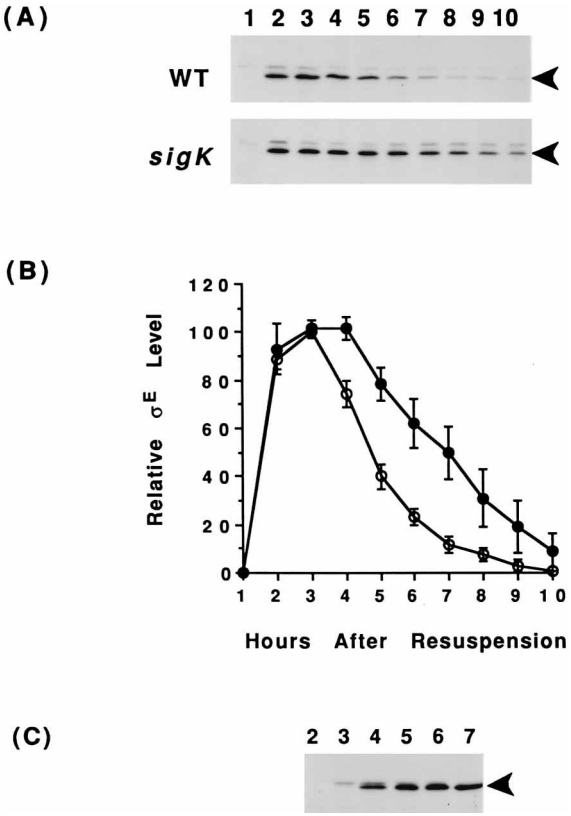

FIG. 2. σ^E persists at a higher level during sporulation of cells defective in σ^K production. Whole-cell extracts were prepared from wild-type (PY79) and *sigK* mutant (BK556) cells collected at the indicated numbers of hours after the onset of sporulation in SM medium. Proteins (5 μg) were fractionated on an SDS–12% polyacrylamide gel and subjected to Western blot analysis with either monoclonal anti-σ^E or polyclonal anti-pro-σ^K antibodies. (A) Levels of σ^E in wild-type (WT) and *sigK* mutant cells. Arrowheads, σ^E signal (the faint signal of lesser mobility most apparent at T_2 is pro-σ^E). (B) Relative amounts of σ^E in wild-type (○) and *sigK* mutant (●) cells during sporulation. The σ^E signals in three experiments with both the wild-type strain and the *sigK* mutant and two experiments with just the wild-type strain were quantified with a computing densitometer. For each experiment, the signal intensities were normalized to the maximum signal in wild-type cells. Points on the graph are averages of the normalized values, and error bars show one standard deviation of the data. (C) Levels of σ^K in wild-type cells. Arrowhead, σ^K signal (the faint signal of lesser mobility first appearing at T_3 is pro-σ^K).

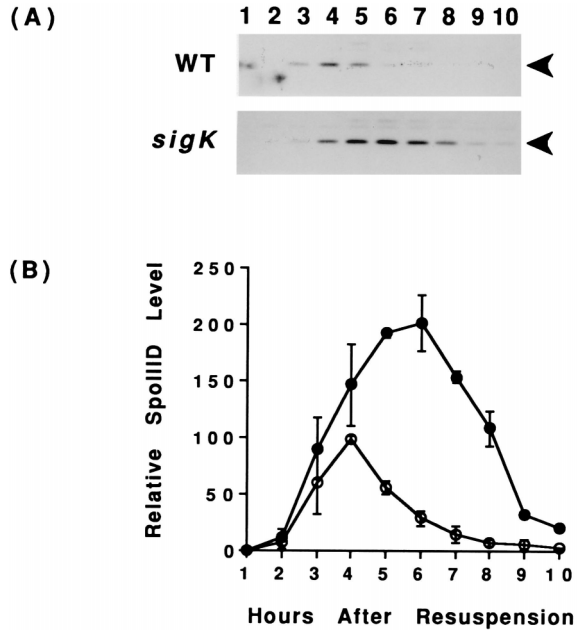

FIG. 3. SpoIIID persists at a higher level during sporulation of cells defective in σ^K production. (A) Proteins (1.7 μg) in the same samples shown in Fig. 2A were fractionated on an SDS–18% polyacrylamide gel and subjected to Western blot analysis with anti-SpoIIID antiserum. Arrowheads, SpoIIID signal. WT, wild type. (B) Relative amounts of SpoIIID in wild-type (PY79 [○]) and *sigK* mutant (BK556 [●]) cells during sporulation. The SpoIIID signals in three experiments were quantified, normalized, and plotted as described in the legend to Fig. 2.

sigK mutant cells (Fig. 1B). Since no substantial difference in the stability of *spoIIID* mRNA was detected, the higher level of *spoIIID* mRNA in sporulating *sigK* mutant cells must be due to increased synthesis of *spoIIID* mRNA. In support of this idea and in agreement with the results of Kunkel et al. (28), we found that a *spoIIID-lacZ* transcriptional fusion is overexpressed by approximately 1.7-fold in *sigK* mutant cells compared to wild-type cells (data not shown).

σ^E level in wild-type and *sigK* mutant cells. Since *spoIIID* is transcribed by σ^E RNA polymerase (28, 49, 52), we reasoned that increased *spoIIID* transcription in *sigK* mutant cells might result from an elevated level of σ^E. We measured the level of σ^E in extracts of wild-type and *sigK* mutant cells using anti-σ^E antibody (30) in Western blot analysis. To facilitate the comparison, the two strains were induced to sporulate in parallel cultures and equal amounts of protein in whole-cell extracts were electrophoresed in the same SDS-polyacrylamide gel. Figure 2A shows that in wild-type cells, σ^E was first detected at T_2 and reached a maximum level by T_3, and the level decreased rapidly thereafter. In *sigK* mutant cells, the σ^E level remained high at T_4, and thereafter its level decreased less rapidly than that in wild-type cells. The experiment was repeated several times, and the Western blot signals were quantitated. Figure 2B shows that, after T_3, σ^E reproducibly persisted at a level in *sigK* mutant cells higher than that in wild-type cells. Between T_5 and T_8, the σ^E level was twofold to fivefold higher in the *sigK* mutant than that in the wild type. Similar results were obtained when other mutants that fail to make σ^K (i.e., cells containing a *spoIIIC94* or a *spoIVCA133* mutation) (35) were tested (data not shown). We also tested mutants (i.e., those containing *spoIIIGΔ1* and *spoIVFΔAB*::*cat*) that produce pro-σ^K but fail to process it to active σ^K (35, 36). Again, similar results were observed (data not shown), indicating the pro-σ^K must be processed to active σ^K in order to accelerate the disappearance of σ^E from sporulating cells. Moreover, as shown in Fig. 2C, processing in wild-type cells causes σ^K to begin accumulating by T_4, which is the earliest time that the σ^E level is lower in wild-type cells than that in *sigK* mutant cells (Fig. 2B). We conclude that the appearance of active σ^K accelerates the disappearance of σ^E during sporulation.

We note that σ^K is not essential for the level of σ^E to decrease, since the σ^E level eventually declines in mutants that fail to make σ^K (Fig. 2B and data not shown). Cell lysis is not the explanation for the decrease in σ^E in the mutants or for the more rapid decrease in σ^E in wild-type cells. Although a small amount of cell lysis began to occur after T_7 in both the wild-type and the mutant cultures, the ability to recover protein from sedimented cells never varied by more than 10% during the course of our experiments.

We also measured SpoIIID levels in most of the samples used in the experiments summarized in Fig. 2B. Figure 3A shows the results for the same samples used in the experiment shown in Fig. 2A. Figure 3B shows quantitation of several experiments. SpoIIID accumulated by T_5 to a level in *sigK*

mutant cells that was twofold higher than that in wild-type cells. The level of SpoIIID in the wild-type strain decreased threefold by T_6, while in the *sigK* mutant the SpoIIID level remained high until T_6 and then declined thereafter. Thus, the levels of both SpoIIID and σ^E are significantly higher in *sigK* mutant cells than in wild-type cells between T_4 and T_8 of sporulation. The absence of σ^K has a larger effect on the SpoIIID level than on the σ^E level (compare Fig. 2B and 3B). This difference might be explained by the fact that σ^E RNA polymerase acts enzymatically to increase *spoIIID* transcription and/or by the observation that SpoIIID positively autoregulates *spoIIID* transcription (23, 28, 49, 52). Thus, a relatively small effect on σ^E could lead to a larger effect on SpoIIID. Clearly, σ^K negatively regulates the σ^E level during sporulation, providing a simple explanation for the negative effect of σ^K on the SpoIIID level.

σ^E level in cells that produce σ^K earlier. It was shown previously that earlier production of σ^K during sporulation resulted in less accumulation of SpoIIID and earlier disappearance of SpoIIID (9). To examine whether these effects might also be explained by a negative effect of σ^K on σ^E, we monitored the level of σ^E in *spoIVCBΔ19* mutant cells. In these cells, codons 2 through 20 of *sigK*, which encode the N-terminal prosequence of pro-σ^K, are missing, resulting in production of active σ^K 1 to 2 h earlier than normal (Fig. 4A) (6, 9). As documented in Fig. 4B and C, the maximum level of σ^E in *spoIVCBΔ19* mutant cells reached only about 50% of the wild-type maximum. These results support the idea that the appearance of σ^K negatively regulates the σ^E level during sporulation.

Turnover of pro-σ^E and σ^E in wild-type and *sigK* mutant cells. σ^K might negatively regulate the σ^E level by destabilizing σ^E, possibly by directly competing with σ^E for core RNA polymerase. It has been suggested that σ^E is unstable in cells when it is not bound to core RNA polymerase (21). A complication in measuring the stability of σ^E is that it is generated from pro-σ^E by proteolytic processing (30). However, since processing of pro-σ^E occurs normally in *sigK* mutant cells (Fig. 2A), we reasoned that a comparison of the total amounts of pro-σ^E and σ^E remaining at different times after pulse-labeling of *sigK* mutant cells and wild-type cells should reveal a difference in σ^E stability, if it exists. Sporulating wild-type and *sigK* mutant cells were pulse-labeled at T_3 with [^{35}S]methionine and chased with an excess amount of unlabeled methionine. We chose T_3 to perform the labeling because during the subsequent hours of sporulation large differences in the levels of σ^E between wild-type and *sigK* mutant cells were observed (Fig. 2B). Samples were collected every half hour after the pulse-labeling, and pro-σ^E and σ^E in crude cell extracts were immunoprecipitated with monoclonal anti-σ^E antibody. The pro-σ^E and σ^E signals were revealed by SDS-PAGE and fluorography (Fig. 5A). The [^{35}S]methionine was first incorporated into pro-σ^E through protein synthesis and then appeared as σ^E upon proteolytic cleavage of the N-terminal sequence from pro-σ^E. A small portion of the pro-σ^E had already been processed into σ^E at the end of the 5 min of pulse-labeling (labeled 0 min in Fig. 5A). Upon incubation, the ^{35}S label was chased into σ^E, and eventually σ^E was degraded. Figure 5B shows that the decay rate of pro-σ^E plus σ^E was similar in wild-type and *sigK* mutant cells, as judged by the quantification of the combined signal intensities of pro-σ^E and σ^E. When the experiment was repeated and samples were collected at 45-min intervals after pulse-labeling, again no substantial difference between wild-type and *sigK* mutant cells was observed (data not shown). Therefore, destabilization of σ^E upon the appearance of σ^K cannot explain the level of σ^E in wild-type cells being lower than that in *sigK* mutant cells at T_4 to T_5 of sporulation (Fig. 2).

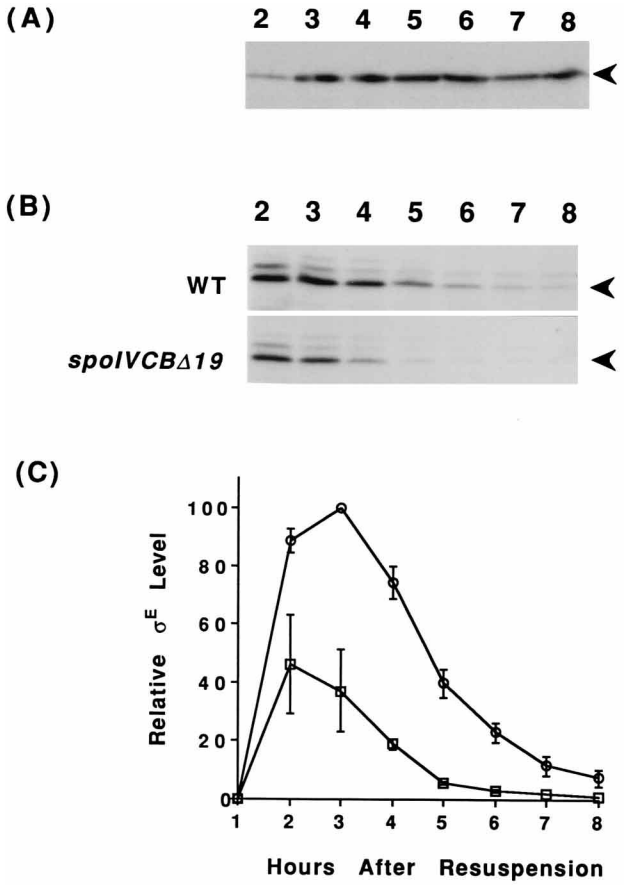

FIG. 4. σ^E disappears earlier during sporulation of cells that produce σ^K earlier than normal. Whole-cell extracts were prepared from wild-type (PY79) and *spoIVCBΔ19* mutant (VO48) cells collected at the indicated numbers of hours after the onset of sporulation in SM medium. Proteins (5 μg) were fractionated on an SDS–12% polyacrylamide gel and subjected to Western blot analysis with either monoclonal anti-σ^E or polyclonal anti-pro-σ^K antibodies. (A) The level of σ^K in the *spoIVCBΔ19* mutant. Arrowhead, σ^K signal. (B) Levels of σ^E in wild-type (WT) and *spoIVCBΔ19* mutant cells. Arrowheads, σ^E signal (the faint signal of lesser mobility most apparent at T_2 is pro-σ^E). (C) Relative amounts of σ^E in wild-type (○) and *spoIVCBΔ19* (□) cells during sporulation. For the wild-type strain, the data shown in Fig. 2B are also shown here. In three of the experiments with wild-type cells, the *spoIVCBΔ19* mutant was induced to sporulate in a parallel culture. The σ^E signals were quantified, normalized, and plotted as described in the legend to Fig. 2.

Expression of a *sigE-lacZ* transcriptional fusion in wild-type cells, *sigK* mutant cells, and cells producing σ^K earlier. Since σ^K did not appear to affect the stability of σ^E, we tested the possibility that σ^K may affect the transcription of the *sigE* gene that encodes pro-σ^E. *sigE* (also called *spoIIGB*) is the second gene in the *spoIIG* operon (22, 24). The first gene of the operon, *spoIIGA*, encodes a putative protease that processes pro-σ^E to σ^E (16, 41, 50). First, we tried to directly compare the levels of *sigE* mRNA in sporulating wild-type and *sigK* mutant cells by Northern blot analysis. In agreement with a previous report (24), we found that *sigE* mRNA was unstable and subject to processing or breakdown. Despite the difficulty in detecting *sigE* mRNA, we noticed that slightly more *sigE* mRNA appeared to be present in *sigK* mutant cells than that in wild-type cells at T_3 and later times during development (data not shown). We then examined expression of a *sigE-lacZ* transcriptional fusion as a simple, albeit indirect, measure of *sigE* transcription. We introduced a *sigE-lacZ* transcriptional fusion

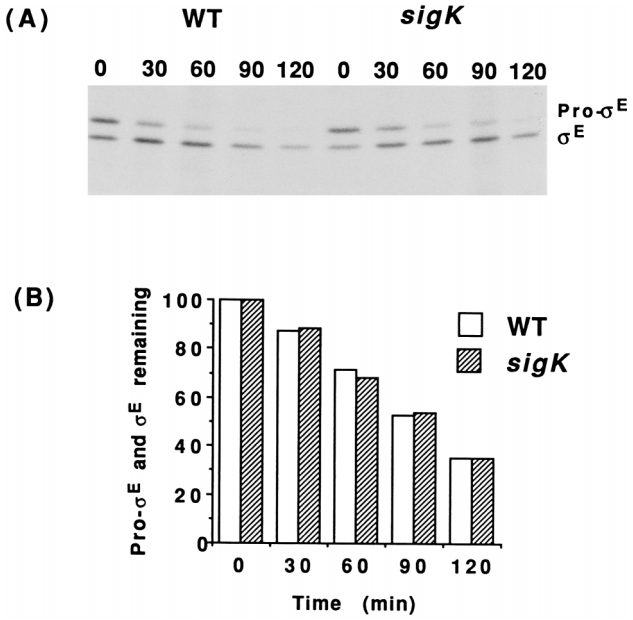

FIG. 5. The production of σ^K does not alter the stability of pro-σ^E and σ^E. (A) Wild-type (PY79) and *sigK* mutant (BK556) cells were labeled at the third hour after the onset of sporulation in SM medium with [^{35}S]methionine for 5 min and chased with excess amounts of unlabeled methionine and cysteine. Cells were collected immediately and at the indicated numbers of minutes following pulse-labeling. Whole-cell extracts were prepared, and pro-σ^E and σ^E were immunoprecipitated with monoclonal anti-σ^E antibody. Immunoprecipitates from 300 μl of the sporulating cell culture were separated by SDS-PAGE and detected by fluorography. (B) Pro-σ^E and σ^E were quantified with a PhosphorImager and plotted as percentages relative to the levels immediately after pulse-labeling. WT, wild type.

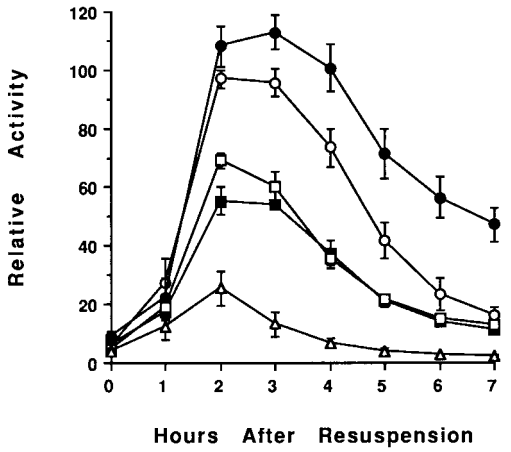

FIG. 6. The effects of altered σ^K production on *sigE-lacZ* expression are similar to the effects on the σ^E level. Wild-type (PY79 [○]) cells, *sigK* mutant (BK556 [●]) cells, and *spoIVCBΔ19* (VO48 [■]), *bofB8* (SC776 [□]), and P_{spac}-P_{sigK}-*sigKΔ19* (BZ536 [△]) cells that produce σ^K earlier than normal were lysogenized with phage SPβ::*sigE-lacZ*, and the resulting strains were induced to sporulate in SM medium. Samples were collected at the indicated times following the initiation of sporulation and assayed for β-galactosidase activity. Experiments were performed at least twice for each strain. For each experiment, the specific activities were normalized to the maximum specific activity in wild-type cells (typically 70 U). Points on the graph are averages of the normalized values, and error bars show one standard deviation of the data.

carried on an SPβ phage (24), via specialized transduction, into the chromosomes of wild-type cells, *sigK* (*spoIVCB23*) mutant cells, and mutants (carrying *spoIVCBΔ19*, *bofB8*, or P_{spac}-P_{sigK}-*sigKΔ19*) that produce active σ^K earlier than normal. The *bofB8* mutant, like the *spoIVCBΔ19* mutant, produces active σ^K about 1 h earlier than normal because processing of pro-σ^K is uncoupled from its normal dependence on a signal from the forespore compartment (6). The P_{spac}-P_{sigK}-*sigKΔ19* mutant contains in its chromosome the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter, *spac*, fused to a copy of the *sigK* gene (*sigKΔ19*) that permits production of active σ^K without the need for the site-specific recombination event that normally joins the two parts (i.e., *spoIVCB* and *spoIIIC*) of the *sigK* gene and without the need for processing (40). Thus, P_{spac}-P_{sigK}-*sigKΔ19* cells produce σ^K upon the addition of IPTG (11, 40). *sigE-lacZ* expression was highest in *sigK* mutant cells, lower in wild-type cells, even lower in *spoIVCBΔ19* and *bofB8* mutant cells, and lowest in P_{spac}-P_{sigK}-*sigKΔ19* cells, as judged by the intensity of blue of colonies sporulating on DSM agar containing X-Gal and IPTG (data not shown). In agreement with these qualitative results were the results of quantitative β-galactosidase assays of cells sporulating in SM liquid. Figure 6 shows that in wild-type cells, *sigE*-directed β-galactosidase activity increased at T_1, reached its peak level at T_2 or T_3, and decreased thereafter. In *sigK* mutant cells, β-galactosidase activity rose to a slightly higher level and remained higher late in sporulation. β-Galactosidase activity was reduced in *spoIVCBΔ19* and *bofB8* mutant cells that produce σ^K about 1 h earlier than normal. The effects on *sigE-lacZ* expression in the *sigK* mutant and the *spoIVCBΔ19* mutant were similar to the effects on the σ^E level (Fig. 2 and 4). When production of σ^K was induced 30 min before cells were resuspended to initiate sporulation, *sigE-lacZ* expression was even lower (Fig. 6). Taken together, these results suggest that σ^K negatively regulates the σ^E level by affecting the transcription of *sigE*.

DISCUSSION

We have demonstrated that σ^K negatively regulates the level of σ^E during sporulation. In wild-type cells, the level of σ^E begins to decrease when active σ^K begins to accumulate (Fig. 2). In mutants defective in σ^K production, σ^E persists at an elevated level for several hours (Fig. 2 and data not shown). In cells engineered to produce σ^K earlier than normal, twofold less σ^E than that in wild-type cells accumulates (Fig. 4). A similar pattern of effects in *sigK* mutant cells and cells prematurely expressing σ^K is observed for expression of a *sigE-lacZ* fusion (Fig. 6), suggesting that σ^K exerts its negative effect at the level of *sigE* transcription.

The finding that σ^K negatively regulates σ^E provides a simple explanation for the previous observation that σ^K negatively regulates SpoIIID (9). As depicted in Fig. 7, σ^E RNA polymerase transcribes the *spoIIID* gene (28, 49, 52). A decrease in the σ^E level brought about by a negative effect of σ^K on *sigE* transcription (Fig. 7) would reduce the synthesis of *spoIIID* mRNA, assuming that σ^E RNA polymerase becomes limiting for *spoIIID* transcription. It seems likely that *spoIIID* transcription is limited by the availability of σ^E, because earlier production of σ^K reduces the σ^E level (Fig. 4), and the level of SpoIIID is likewise reduced (9). Conversely, the failure to make σ^K results in an elevated σ^E level beginning at T_4 of sporulation (Fig. 2), and the level of SpoIIID is also elevated (Fig. 3). We found no evidence that σ^K affects *spoIIID* expression at the level of mRNA stability (Fig. 1). Also, there is no evidence that σ^K regulates the SpoIIID level via a posttranscriptional mechanism. The difference in the SpoIIID protein level between wild-type and *sigK* mutant cells (Fig. 3) is similar

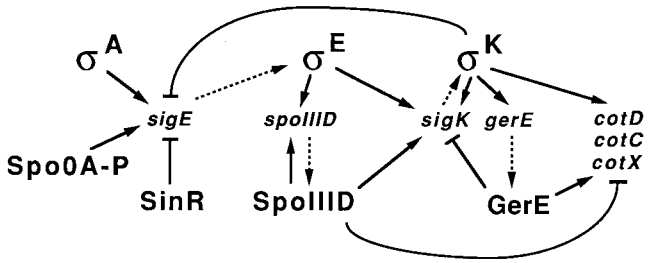

FIG. 7. Model for gene regulation in the mother cell cascade. Dashed lines with arrowheads, gene-to-product relationships; arrows and lines with barred ends, positive and negative effects, respectively, on expression. The hallmark of initiation of sporulation is an increase in the level of Spo0A-P. Spo0A-P activates transcription of *sigE* by σ^A RNA polymerase. SinR directly or indirectly inhibits *sigE* expression. σ^E RNA polymerase transcribes *spoIIID*. SpoIIID regulates genes in both the σ^E and σ^K regulons. Among them, it activates *sigK* transcription and represses transcription of certain *cot* genes. Transcription of *sigK* is directed first by σ^E and then by its own gene product, σ^K. The *cotD*, *-C*, and *-X* genes are transcribed by σ^K RNA polymerase. σ^K negatively regulates *spoIIID* and the entire σ^E regulon by negatively regulating *sigE* transcription. A diminished SpoIIID level allows the previously repressed *cot* genes to be transcribed. GerE is a gene product of the σ^K regulon. It represses transcription of *sigK*, forming another feedback loop in the mother cell cascade of gene expression. GerE also activates transcription of the *cotD*, *-C*, and *-X* genes, reinforcing the switch of the mother cell gene expression pattern initiated by the decrease in the level of SpoIIID.

to the difference in the *spoIIID* mRNA level (9) (data not shown). In addition, the pattern of overaccumulation of β-galactosidase activity from a *spoIIID-lacZ* transcriptional fusion in sporulating *sigK* mutant cells (28) (data not shown) was similar to the pattern of overaccumulation of SpoIIID (Fig. 3). Therefore, we propose that σ^K directly or indirectly inhibits *sigE* transcription, reducing synthesis of σ^E, which in turn reduces transcription of *spoIIID*, and, as the level of SpoIIID declines, its repressive effect on σ^K-dependent genes such as *cotD*, *cotX*, and *cotC* is relieved (9, 10, 19, 56, 58) (Fig. 7).

Transcription of *sigE* is carried out by σ^A RNA polymerase and requires Spo0A phosphate (25, 47) (Fig. 7). σ^A is the major sigma factor present in growing cells, in which it directs transcription of most genes (12). Spo0A is also present in growing cells, and an increase in the level of phosphorylated Spo0A (Spo0A-P) initiates sporulation gene expression, including directly activating *sigE* transcription by σ^A RNA polymerase, in response to nutritional, extracellular, and cell cycle signals (1, 20). Transcription of *sigE* is also subject to negative control by SinR (37, 38) (Fig. 7). We are currently trying to determine whether σ^K exerts its negative effect on *sigE* transcription by affecting σ^A, Spo0A-P, or SinR.

The negative effect of σ^K on *sigE* transcription may explain why a *sigE-lacZ* fusion is overexpressed in *sigE* mutant cells (24). Since the *sigE* mutant fails to make σ^K, the negative feedback on *sigE* transcription would not occur, resulting in *sigE* overexpression. Similarly, the elevated level of σ^E found in *sigK* mutant cells might cause overexpression of other σ^E-dependent genes in addition to *spoIIID*. The promoter of *spoIID* is a well-known example of a σ^E-dependent promoter that is independent of SpoIIID for transcription (4, 44). We found that *spoIID-lacZ* is overexpressed in *sigK* mutant cells (data not shown).

The negative effect of σ^K on σ^E and SpoIIID is not the only example of a feedback loop in the cascade of transcription factors controlling mother cell gene expression. As illustrated in Fig. 7, σ^K RNA polymerase transcribes the *gerE* gene and GerE limits the σ^K level by repressing *sigK* transcription (19, 58). It was attractive to think that in addition to repressing *sigK* transcription, GerE might repress the transcription of *sigE* and/or *spoIIID*. However, expression of *sigE-lacZ* and *spoIIID-lacZ* transcriptional fusions is indistinguishable in wild-type and *gerE* mutant cells (data not shown).

The finding that σ^K negatively regulates *sigE* transcription provides an alternative to the model that each subsequent σ in a cascade competes more effectively for a limiting amount of core RNA polymerase (33). In vitro studies with phage σ factors involved in cascade regulation support the direct σ competition model in some cases (2, 18), but not in others (53). Recently, Hicks and Grossman (15) presented in vivo experiments that suggest that σ^A competes with σ^H for binding to core RNA polymerase. If σ^K could outcompete σ^E for core binding, it seemed likely that the appearance of σ^K in cells would destabilize σ^E, since it had been suggested that free σ^E is unstable (21). However, σ^K did not affect the stability of σ^E (Fig. 5).

In the σ cascade controlling *B. subtilis* sporulation gene expression, each σ is either made as an inactive precursor or is initially held inactive by an anti-σ factor (12, 34). This ensures that later-acting σ factors accumulate sufficiently before negatively regulating earlier-acting σ factors that control their synthesis. Regulation of σ factor activity also appears to couple the program of gene expression in the mother cell and forespore during *B. subtilis* sporulation (26, 34). For example, proteolytic processing of inactive pro-σ^K to active σ^K in the mother cell is governed by a signal transduction pathway that emanates from the forespore and may depend on a morphological feature of the developing sporangium (5–7, 35). In this case, the primary event responding to morphological and/or cell-cell signals is pro-σ^K processing. Loss of σ^E and SpoIIID is a secondary event brought about by the negative effect of σ^K on *sigE* transcription. In contrast, loss of a transcription factor from cells due to secretion is the primary event regulated by morphological cues or cell-cell interactions in a few examples that have emerged recently (17, 42, 57). These examples highlight the importance of considering the disappearance of existing transcription factors, as well as the appearance of new ones, during adaptive processes.

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