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Sporulation

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Lee Kroos
Major professor

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CHARACTERIZATION OF THE ROLE OF THE SPOIIID SWITCH PROTEIN DURING BACILLUS SUBTILIS SPORULATION

By

Richard Brott Halberg

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ABSTRACT

CHARACTERIZATION OF THE ROLE OF THE SPOIIID SWITCH PROTEIN DURING BACILLUS SUBTILIS SPORULATION

By

Richard Brott Halberg

A fundamental problem in biology is understanding how gene expression is regulated temporally and spatially during the development of living organisms. A particularly tractable system to address this problem is the gram-positive bacterium *Bacillus subtilis*, because it is amenable to both genetic and biochemical approaches.

Under conditions of nutrient deprivation, *B. subtilis* undergoes a series of morphological changes which culminate in the formation of a spore. The first easily-observed morphological structure is an asymetrically-positioned septum, which divides the bacterium into two compartments, the mother cell and the forespore. Both of these compartments receive a copy of the genome, but they realize alternative developmental fates because gene expression is regulated temporally and spatially. Three key regulators of transcription in the mother cell are SpoIIID, σ^E, and σ^K.

The research presented in this dissertation is focused on characterizing the role of SpoIIID during sporulation. The ability of SpoIIID to affect the transcription of σ^E- and σ^K-dependent genes was tested *in vitro*. SpoIIID activates and represses transcription by both forms of polymerase. SpoIIID appears to have these effects by binding to specific sequences in the -35 region of σ^E- and σ^K-dependent genes, as evidenced by DNase I footprinting. A comparison of the sequences in the protected regions revealed a putative consensus sequence for binding of SpoIIID.

The fate of SpoIIID during sporulation was determined by Western blot analysis. The level of this protein decreases sharply during the late stages. The decrease appears to be controlled by two independent mechanisms: one acts at the level of *spoIIID* mRNA synthesis and/or stability, while the other involves the conversion of SpoIIID to a less stable 9 kDa form. The conversion appears to involve the removal of 7 amino acids from the C-terminus of SpoIIID, based on N-terminal sequencing and mass analysis.

The proper level of SpoIIID is crucial for normal sporulation. Cells engineered to produce an elevated level of SpoIIID throughout sporulation produced fewer heat-resistant spores. This effect appears to result from a reduction in σ^K production and σ^K-dependent gene expression.

This dissertation is dedicated with love and respect to

Sara
Tim
Emily
Mom
Dad
Grandma
Joe
Lois
Dave
Carol
Bob
Kathy
Kevin
Theresa
Bob
Margie
Dean
Jane
Steve
Karen
Doober
Jane

and

Friends

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When considering graduate school, I never believed that a person could be a Ph.D. candidate and have a family at the same time. Now, I can not imagine one without the other. I would like to thank my wonderful wife, Sara, for her love and support, and two children, Tim and Emily, for their warm smiles and innocent ways. I would also like to thank my parents for always encouraging me to pursue my dreams.

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Lee and his wife, Mary, have been very supportive during life's challenges, especially, the adoption of Tim and the birth of Emily. Lee and Mary are tremedous friends that I will **greatly** miss.

I have also had the opportunity to work with great friends and collegues throughout my research career. I would like to thank: Ted for taking me under his wing and sharing numerous dinners with Sara and me, Kevin for constantly arguing with me and making sure I saw George Bush in 1988, Dave for arguing with Kevin and showing me how golf should be played, Mark Sutton for his mutual interest in Mike Dikta and the Chicago Bears, Mark Sinton for computer advice and sharing risky experiences, Eugene for his expertise with MALDI mass spectroscopy and his fatherly advice, Sara for numerous meals and sharing Brian with me, Brian for sharing the joys of fatherhood and fighting numerous battles, Jamie for just being Jamie, Sijie for making scientific meetings enjoyable and my first experiences with cell death, Monica for her great energy and banana spilt bread, Makda for her methodically ways and all encompassing knowledge of camels, Hiroshi for doing all the experiments that I should of done and his multitude of hairstyles, Bin for doing more of the experiments I should have done and his ability to acquire free samples, and Janine for housing tips and humerous cat stories.

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I would like to thank Joe, Melanie, and Colleen for sequencing the 9 kDa protein, preparing primers, and advice and Diane, Julie, Mary, Vickie, and Carol for getting me out of numerous dilemas. I would also like to thank George and Nick for their friendship.

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LIST OF ABBREVIATIONS

ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
bp	base pair
BRL	Bethesda Research Laboratories
BSA	bovine serum albumin
CTP	cytosine-5'-triphosphate
Da	dalton
DNA	deoxyribonucleic acid
DPA	dipicolinic acid
DTT	dithiothreitol
EDF-A	extracellular differentiation factor
EDTA	(ethylenedinitrilo)tetraacetic acid
GDP	guanosine-5'-diphosphate
GTP	guanosine-5'-triphosphate
HCl	hydrochloric acid
IPTG	isopropyl ß-D thiogalactopyranoside
kb	kilobases
kDa	kilodalton
LB	Luria-Bertani
MALDI	matrix-assisted laser desorption ionization

M	molar
mM	millimolar
$MgCl_2$	magnesium chloride
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
ng	nanogram
O.D.	optical density
ONPG	*o*-nitrophenol-ß-D-galactoside
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
pmole	picomole
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
SDS	sodium dodecylsulfate
SM	Sterlini-Mandelstam
T_x	x hours after the onset of sporulation
TFA	trifluoroacetic acid
µg	microgram
µl	microliter
UTP	uracil-5′-triphosphate
v/v	volume per volume
w/v	weight per volume

INTRODUCTION

Under conditions of nutrient deprivation, *Bacillus subtilis* undergoes a series of morphological changes which culminate in the formation of a spore. The first easily observed morphological structure is an asymmetrically positioned septum, which divides the bacterium into two compartments, the mother cell and the forespore. Both of these compartments receive a copy of the genome, but they realize alternative developmental fates because gene expression is regulated temporally and spatially. Regulation in the mother cell involves two, small DNA-binding proteins, SpoIIID and GerE, as well as two sigma subunits of RNA polymerase, σ^E and σ^K.

The experiments in Chapter II demonstrate that SpoIIID activates and represses transcription by both the σ^E and σ^K forms of RNA polymerase. SpoIIID appears to have these effects by binding specific sequences in the promoter regions of σ^E- and σ^K-dependent genes. This work was submitted to the *Journal of Molecular Biology*.

Based on the observation that SpoIIID can activate and repress transcription by σ^K RNA polymerase, it was proposed that inactivation and/or sequestering of SpoIIID would establish a switch in the pattern of gene expression. The experiments in Chapter III focus on testing this model. The level of SpoIIID does decrease sharply at the appropriate time to establish such a switch. This decrease is, in part, due to the production of active σ^K, which leads to reduced synthesis and/or stability of *spoIIID* mRNA. This work was published in the *Journal of Molecular Biology*.

The decrease in the level of *spoIIID* mRNA is paralleled by a decrease in the level of SpoIIID, suggesting that a mechanism for degrading SpoIIID exists in sporulating *B. subtilis*. The experiments in Chapter IV provide evidence that SpoIIID is converted to a less stable 9 kDa form by removing 7 amino acids from its C-terminus. This conversion is developmentally regulated. The mass spectral data presented in this chapter was obtained through a collaboration with E. Zaluzec and D. Gage at Michigan State University.

The proper level of SpoIIID is crucial for normal development. The experiments in Chapter V demonstrate that overproducing SpoIIID significantly reduces the production of heat-resistant spores. This effect appears to result from a reduction in σ^K production and σ^K-dependent gene expression. The experiments in this chapter also provide evidence that

SpoIIID represses *cotD* expression by σ^K RNA polymerase *in vivo*. V. Oke at Harvard University provided the strain which permits σ^K to be produced during growth.

The experiments in appendix A demonstrate that the primary translation product of *sigK* is pro-σ^K, an inactive precursor, which is proteolytically-processed to the active form. This work was done by S. Lu, L. Kroos and myself. I was responsible for determining whether pro-σ^K was capable of directing transcription *in vitro*. This work was published in the *Proceedings of the National Academy of Science USA*.

The experiments in appendix B demonstrate that the switch in the mother-cell pattern of gene expression, which is established by the production of σ^K and subsequent decrease in the level of SpoIIID, is reinforced by GerE. This work was a collaboration between L. Zhang, S. Roels, R. Losick at Harvard University and H. Ichikawa, L. Kroos, and myself at Michigan State University. I was responsible for preparing some of the RNA for the primer extension experiments and the preparation of materials used in the *in vitro* transcription assays. This work was published in the *Journal of Molecular Biology*.

CHAPTER I

LITERATURE REVIEW

"To be absolutely certain about something, one must know everything or nothing about it."

Olin Miller

Overview

A fundamental problem in developmental biology is understanding how gene expression is regulated temporally and spatially during the development of living organisms. A particularly tractable system to address this problem is the gram-positive bacterium *Bacillus subtilis*, because it is amenable to both genetic and biochemical approaches.

Under conditions of nutrient deprivation, *B. subtilis* sporulates. This process has been divided into several stages based on the morphological changes which occur (Figure 1; reviewed by Errington, 1993). The first easily observed change is the formation of an asymmetric septum, which divides the bacterium into two compartments, the larger mother cell and the smaller forespore (stage II). Migration of the septum results in the engulfment of the forespore as a free, double-membraned protoplast within the mother cell (stage III). A cell-wall-like material, called cortex, is deposited between the membranes of the forespore (stage IV) and then coat proteins, which are synthesized in the mother cell, are deposited on the outer surface of the forespore (stage V). The spore matures, gaining all of its resistance properties (stage VI), and is released by the lysis of the mother cell (stage VII). This process takes about six to ten hours at 37°C.

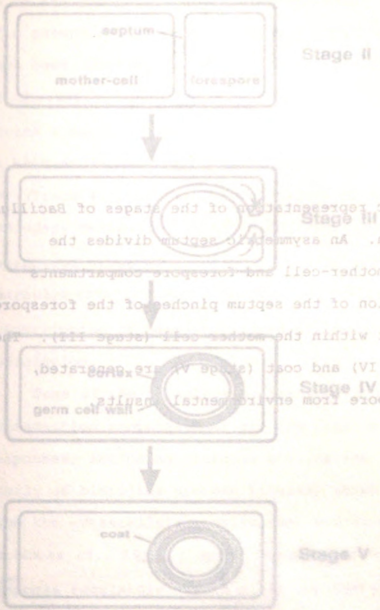
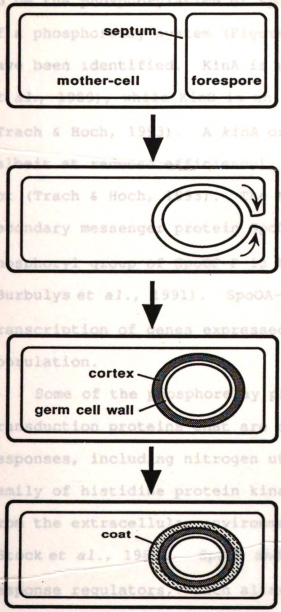

septum
mother-cell
forespore
Stage II
Stage III
cortex
germ cell wall
Stage IV
coat
Stage V

Figure 1. Schematic representation of the stages of *Bacillus subtilis* sporulation. An asymmetric septum divides the bacterium into the mother-cell and forespore compartments (stage II). Migration of the septum pinches of the forespore as a free protoplast within the mother cell (stage III). The spore cortex (stage IV) and coat (stage V) are generated, which protect the spore from environmental insults.

Initiation of sporulation

The key event in the initiation of sporulation appears to be the phosphorylation of SpoOA, which is the end-result of a phosphorelay system (Figure 2; Hoch, 1994). Two kinases have been identified. KinA is a cytoplasmic protein (Perego et al., 1989), while KinB is an integral membrane protein (Trach & Hoch, 1993). A *kinA* or *kinB* mutant sporulates (albeit at reduced efficiency), whereas a double mutant does not (Trach & Hoch, 1993). The target of KinA and KinB is the secondary messenger protein SpoOF (Perego *et al.*, 1989). The phosphoryl group of SpoOF~P is transferred to SpoOA *via* SpoOB (Burbulys *et al.*, 1991). SpoOA~P activates and represses transcription of genes expressed during stationary phase and sporulation.



Some of the phosphorelay proteins are similar to signal transduction proteins that are involved in numerous adaptive responses, including nitrogen utilization. KinA belongs to a family of histidine protein kinases, which receive signals from the extracellular environment and/or the cytoplasm (Stock *et al.*, 1989). SpoOF and SpoOA belong to a family of response regulators, which alter the pattern of gene expression, allowing the bacterium to adapt to its environment (Stock *et al.*, 1989).

The phosphorylation of SpoOA appears to incorporate both

Initiation of sporulation

The key event in the initiation of sporulation appears to be the phosphorylation of Spo0A, which is the end-result of a phosphorelay system (Figure 2; Hoch, 1994). Two kinases have been identified. KinA is a cytoplasmic protein (Perego *et al.*, 1989), while KinB is an integral membrane protein (Trach & Hoch, 1993). A *kinA* or *kinB* mutant sporulates (albeit at reduced efficiency), whereas a double mutant does not (Trach & Hoch, 1993). The target of KinA and KinB is the secondary messenger protein Spo0F (Perego *et al.*, 1989). The phosphoryl group of Spo0F-P is transferred to Spo0A *via* Spo0B (Burbulys *et al.*, 1991). Spo0A-P activates and represses transcription of genes expressed during stationary phase and sporulation.

Some of the phosphorelay proteins are similar to signal transduction proteins that are involved in numerous adaptive responses, including nitrogen utilization. KinA belongs to a family of histidine protein kinases, which receive signals from the extracellular environment and/or the cytoplasm (Stock *et al.*, 1989). Spo0F and Spo0A belong to a family of response regulators, which alter the pattern of gene expression, allowing the bacterium to adapt to its environment (Stock *et al.*, 1989).

The phosphorylation of Spo0A appears to incorporate both

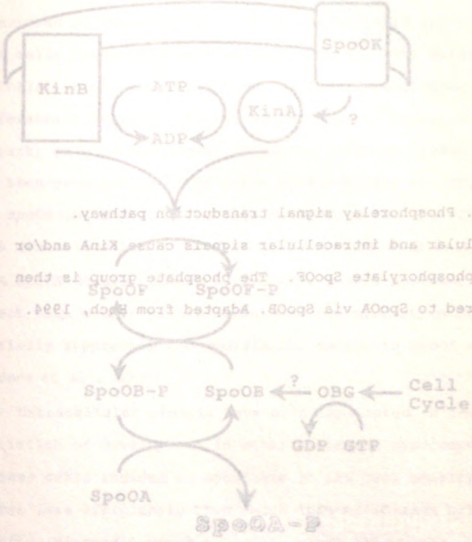
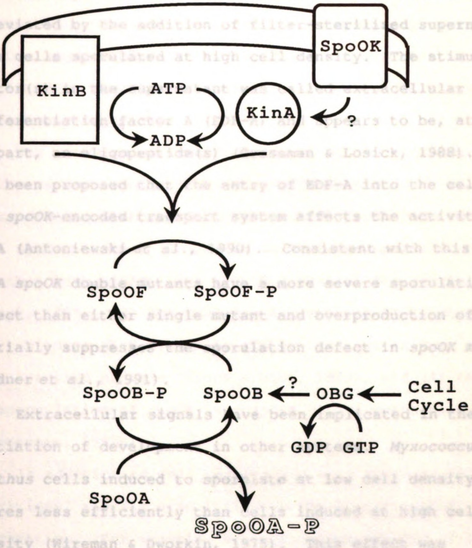

KinB
Spo0K
ATP
KinA
?
ADP
Spo0F
Spo0F-P
Spo0B-P
Spo0B
?
OBG
Cell Cycle
GDP
GTP
Spo0A
Spo0A-P

Figure 2. Phosphorelay signal transduction pathway. Extracellular and intracellular signals cause KinA and/or KinB to phosphorylate SpoOF. The phosphate group is then transferred to SpoOA via SpoOB. Adapted from Hoch, 1994.


SpoOK
KinB
ATP
KinA
?
ADP
SpoOF
SpoOF-P
SpoOB-P
SpoOB
?
OBG
Cell
Cycle
GDP
GTP
SpoOA
SpoOA-P

extracellular and intracellular signals. KinA activity may be regulated by an extracellular signal. Cells induced to sporulate at low cell density form heat-resistant spores about 10^4-fold less efficiently than cells induced at high cell density (Grossman & Losick, 1988). This effect is alleviated by the addition of filter-sterilized supernatant from cells sporulated at high cell density. The stimulatory factor(s) in the supernatant was called extracellular differentiation factor A (EDF-A) and appears to be, at least in part, an oligopeptide(s) (Grossman & Losick, 1988). It has been proposed that the entry of EDF-A into the cell *via* the *spoOK*-encoded transport system affects the activity of KinA (Antoniewski *et al.*, 1990). Consistent with this idea, *kinA spoOK* double mutants have a more severe sporulation defect than either single mutant and overproduction of KinA partially suppresses the sporulation defect in *spoOK* mutants (Rudner *et al.*, 1991).

Extracellular signals have been implicated in the initiation of development in other systems. *Myxococcus xanthus* cells induced to sporulate at low cell density formed spores less efficiently than cells induced at high cell density (Wireman & Dworkin, 1975). This effect was alleviated by the addition of A-factor, which is composed of amino acids, oligopeptides and proteases (Kuspa *et al.*, 1992; Plamann *et al.*, 1992). The role of the proteases appears to be to release amino acids and oligopeptides. The amino acids and oligopeptides may enter the cell by a transport system similar to that encoded by *spoOK*, because two genes at the *sasA* (suppressor of A-signaling defect) locus resemble genes in the *spoOK* operon (H. Kaplan, unpublished data). The entry of amino acids and oligopeptides may affect a signal transduction pathway similar to the phosphorelay system, because *asgA* (a-signalling) encodes a protein that is similar to histidine kinases and response regulators (L. Plamann, unpublished data).

The activity of other components of the phosphorelay system may be regulated by intracellular signals. It has been proposed that Obg links the activity of SpoOB to the cell cycle, because (1) *obg* is in the same operon as *spoOB* (Trach & Hoch, 1989), (2) Obg is essential for growth (Trach & Hoch, 1989), (3) Obg contains a guanosine-5'-triphosphate (GTP) binding site, which is similar to that in Era, an E. coli Ras-like protein (Trach & Hoch, 1989), and (4) the phosphorylation of SpoOA is coupled to DNA synthesis (Ireton & Grossman, 1992b).

In addition to cell density and cell cycle signals, nutritional signals also influence the initiation of sporulation. For example, starvation for sources of carbon, nitrogen, or phosphorous can induce sporulation. The mechanisms for sensing nutritional signals have not been elucidated. However, some experiments suggest GTP (and/or GDP) represents the key effector of the nutritional signals

(Freese *et al.*, 1985). Thus, Obg may incorporate nutritional signals into the phosphorelay system, if it really binds GTP and affects the activity of Spo0B.

Role of Sigma Factors During Sporulation

The temporal and spatial pattern of gene expression during sporulation is established, in part, by the sequential activation of compartment-specific sigma subunits of RNA polymerase.

Forespore-specific sigma factors

spoIIA is a three-cistron operon, consisting of *spoIIAA*, *spoIIAB*, and *spoIIAC* (Fort & Piggot, 1984; Piggot *et al.*, 1984; Errington *et al.*, 1985; Stragier, 1986; Sun *et al.*, 1989). Transcription of *spoIIA* is dependent upon Spo0A-P and σ^H RNA polymerase (Zuber *et al.*, 1989), the earliest acting sporulation-specific form of RNA polymerase (Errington, 1986; Savva & Mandelstam, 1986; Burbulys *et al.*, 1991), and begins prior to septation (Gholamhoseinian & Piggot, 1989). However, the product of *spoIIAC*, σ^F, appears to be active only in the forespore, since ß-galactosidase expressed from a σ^F-dependent *lacZ* fusion is localized to this compartment (Margolis *et al.*, 1991). σ^F activity is regulated by *spoIIAA* and *spoIIAB*. Genetic studies demonstrated that σ^F activity is antagonized by *spoIIAB* and that *spoIIAB* is antagonized by *spoIIAA* (Schmidt *et al.*, 1990). Biochemical studies revealed that SpoIIAB is an anti-sigma factor that binds to σ^F and blocks σ^F-directed transcription. SpoIIAB can also bind to SpoIIAA (Duncan & Losick, 1993). SpoIIAB binding to σ^F is stimulated by adenosine-5'-triphosphate (ATP) and its non-hydrolyzable analogs, while SpoIIAB binding to SpoIIAA is stimulated by adenosine-5'-diphosphate (ADP) (Alper *et al.*, 1994). Based on these observations, it has been proposed that the concentration of ATP decreases and the concentration of ADP increases in the forespore, resulting in SpoIIAB binding SpoIIAA rather than σ^F and thereby σ^F becoming active specifically in the forespore. In contrast, the concentration of ATP remains high relative to ADP in the mother cell, resulting in SpoIIAB remaining bound to σ^F and thereby blocking σ^F-dependent gene expression. Consistent with this idea, the concentration of ATP relative to other adenosine nucleotides decreases dramatically in the forespore during sporulation of *Bacillus megaterium* (Singh *et al.*, 1977).

spoIIIG encodes σ^G (Masuda *et al.*, 1988; Karmazyn-Campelli *et al.*, 1989; Sun *et al.*, 1989), which is required for the transcription of a family of small, acid-soluble proteins that protect the spore DNA from different types of environmental insults (Mason *et al.*, 1988). *spoIIIG* appears to be transcribed by three different mechanisms. Prior to the formation of the asymmetric septum (stage II), *spoIIIG* is transcribed as the third member of the *spoIIG* operon (discussed below) by σ^A RNA polymerase, the vegetative form of RNA polymerase, and SpoOA-P (Masuda *et al.*, 1988; Karmazyn-Campelli *et al.*, 1989). However, this probably does not lead to the production of σ^G, because there is a stem-loop structure located upstream of *spoIIIG* that is predicted to block its translation. After septation, *spoIIIG* is transcribed by σ^F RNA polymerase from a promoter located proximal to the *spoIIIG* ORF (Sun *et al.*, 1989; Schmidt *et al.*, 1990; Partridge *et al.*, 1991). However, this early transcription does not appear to lead immediately to active σ^G, because there is only a very low level of ß-galactosidase expressed from fusions between σ^G-dependent promoters and *lacZ* at this time (Mason *et al.*, 1988). It has been proposed that like σ^F, σ^G is held inactive by an anti-sigma factor. In fact, genetic and biochemical studies suggest that SpoIIAB inhibits σ^G activity (Rather & Moran, 1988; Kirchman *et al.*, 1993). If SpoIIAB does repress both σ^F and σ^G activity, then its repression of these sigma factors must be relieved by different mechanisms, because σ^F and σ^G are activated sequentially. After engulfment of the forespore by migration of the sporulation septum (stage III), σ^G becomes active and directs the transcription of its own gene (Karmazyn-Campelli *et al.*, 1989) as well as other genes (Mason *et al.*, 1988). Autoregulation provides a burst of *spoIIIG* expression exclusively in the forespore.

Mother-cell-specific sigma factors

spoIIG locus contains two genes, *spoIIGA* and *spoIIGB*, involved in the production of active σ^E, which is required for the transcription of genes that are involved in engulfment, cortex formation, and coat formation (Trempy *et al.*, 1985a; Trempy *et al.*, 1985b; Jonas *et al.*, 1988; Stragier *et al.*, 1988). Transcription of *spoIIG* by σ^A RNA polymerase and SpoOA-P begins prior to formation of the asymmetric septum (Kenney *et al.*, 1988; Kenney *et al.*, 1989; Satola *et al.*, 1991; Satola *et al.*, 1992). However, the product of *spoIIGB*, σ^E, appears to be active only in the mother cell, because ß-galactosidase activity expressed from a σ^E-dependent *lacZ* fusion is localized to the mother cell (Driks & Losick, 1991). σ^E is synthesized as an inactive precursor, pro-σ^E, with an additional 27-29 amino acids at its N-terminus (LaBell *et al.*, 1987). Proteolytic processing of pro-σ^E to σ^E is dependent upon *spoIIGA*, which encodes a protein that contains some sequences conserved in aspartic proteases (Jonas *et al.*, 1988; Stragier *et al.*, 1988). Processing of pro-σ^E to σ^E appears to occur only after septation (stage II).

sigK encodes σ^K (Stragier *et al.*, 1989), which is required for the transcription of several spore coat proteins that protect the spore from environmental insults (Donovan *et al.*, 1987; Sandman *et al.*, 1988; Zheng & Losick, 1990; Cutting *et al.*, 1991c). *sigK* is a composite gene that is generated by joining *spoIVCB*, encoding the N-terminal portion of σ^K, to *spoIIIC*, encoding the C-terminal portion of σ^K (Stragier *et al.*, 1989). This event occurs exclusively in the mother cell, because it is dependent upon two genes, *spoIVCA* and *spoIIID* (Kunkel *et al.*, 1990), which are transcribed by σ^E RNA polymerase (Tatti *et al.*, 1991; Sato *et al.*, 1994). *spoIVCA* encodes the putative recombinase (Kunkel *et al.*, 1990; Sato *et al.*, 1990), while spoIIID encodes a DNA-binding protein (Kroos *et al.*, 1989; Kunkel *et al.*, 1989; Stevens & Errington, 1990). *spoIVCA* transcription by σ^E RNA polymerase is stimulated by SpoIIID (Sato *et al.*, 1994; Chapter II).

In addition to affecting *spoIVCA* transcription, SpoIIID stimulates *sigK* transcription by σ^E RNA polymerase (Kunkel *et al.*, 1988; Chapter II). However, this does not lead immediately to active σ^K, because like σ^E, σ^K is synthesized as an inactive precursor, in this case with an additional 20 amino acids at its N-terminus (Kroos *et al.*, 1989; Stragier *et al.*, 1989; Lu *et al.*, 1990). Proteolytic processing is dependent upon several sporulation genes (discussed below) and does not occur until after the completion of forespore engulfment (stage III). σ^K directs the transcription of its own gene (Kunkel *et al.*, 1988; Kroos *et al.*, 1989) as well as other genes (Zheng & Losick, 1990; Cutting *et al.*, 1991c). σ^K-dependent transcription is affected by SpoIIID and by another small DNA-binding protein, GerE (discussed below).

Specific proteolysis is a regulatory mechanism used to control gene expression in other organisms. For example, the p50 subunit of NF-kappa-B, a transcription factor that affects the expression of genes involved in immune function, inflammation, and cellular growth, is generated by removing the C-terminal portion of p105 through an ATP-dependent proteolytic pathway (Fan & Maniatas, 1991). The p50 subunit binds DNA, whereas p105 does not (Ghosh, 1990; Kieran, 1990), indicating that the C-terminus interferes with the interaction between the DNA-binding domain (located at the N-terminus) and DNA. Similarly, the additional 20 amino acids at the N-terminus of pro-σ^K reduces the affinity of this protein for its cognate promoters (Dombroski *et al.*, 1993).

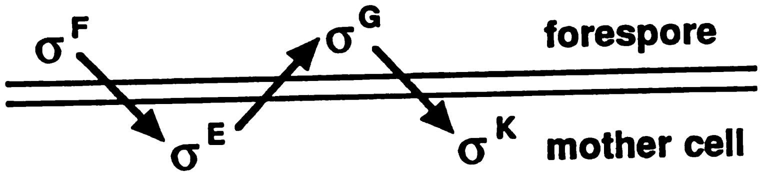
Intercompartmental Coupling of Gene Expression in Both Compartments

Although transcription is driven by forespore- and mother-cell-specific sigma factors, gene expression in each compartment is coupled to events that occur in the other compartment, because σ^F is required for the activation of σ^E, σ^E is required for the activation of σ^G, and σ^G is required for the activation of σ^K (Figure 3). The mechanisms for intercompartmental coupling are still unclear. However, recent studies have provided some insight (reveiwed by Kroos and Cutting, 1994).

σ^F and σ^E

It has been proposed that σ^F-directed gene expression in the forespore leads to a modification of the sporulation septum that triggers pro-σ^E processing in the mother cell (Higgins & Piggot, 1992; Losick & Stragier, 1992). Consistent with this hypothesis are two observations. First, σ^F activity appears to be required to remove a thin

Figure 3. Criss-cross activation of sigma factors during sporulation. The double line represents the membrane separating the forespore and mother-cell compartments. Adapted from Kroos and Cutting, 1994.


σ F
σ E
σ G
σ K
forespore
mother cell

layer of peptidoglycan that initially forms in the sporulation septum (Illing & Errington, 1991a; Higgins & Piggot, 1992). Second, SpoIIGA (the putative processing enzyme or regulator of processing) is predicted to contain five membrane-spanning domains and appears to be an integral membrane protein (Peters & Haldenwang, 1991), so it may be in position to sense a morphological change in the sporulation septum. Thus, processing of pro-σ^E to σ^E may couple gene expression in the mother cell to gene expression in the forespore and formation of the sporulation septum. However, other experiments indicate that pro-σ^E is processed to σ^E in both compartments (Carlson & Haldenwang, 1989; Kirchman *et al.*, 1993). The relative amounts of pro-σ^E and σ^E in the two compartments are still being debated. Based on these observations, it was proposed that σ^F directs the transcription of two genes: one encoding a product required for processing and the other encoding a product that inhibits σ^E activity in the forespore (Stragier *et al.*, 1994).

σ^E and σ^G

σ^E directs the transcription of *spoIID* and *spoIIIA* (Rong *et al.*, 1986; Driks & Losick, 1991; Illing & Errington, 1991b), which are both required for the activation of σ^G.

spoIID encodes a 37 kDa protein, which resembles a modifier of a cell wall hydrolytic enzyme (Kuroda *et al.*, 1992; Lazarevic *et al.*, 1992). It has been proposed that this protein may play a role in the release of the forespore in the mother cell (Kuroda *et al.*, 1992; Lazarevic *et al.*, 1992). *spoIIIA* is a seven-cistron operon. All of these genes appear to encode proteins with membrane spanning domains (P. Stagier, unpublished data). It has been proposed that these proteins reside in the outer membrane of the forespore and affect σ^G-dependent transcription by affecting the stability of SpoIIAB (Kirchman *et al.*, 1993). Consistent with this idea, the level of SpoIIAB remained high in both compartments during the development of *spoIIIA* mutant cells, but was greatly reduced in the forespore during the development of wild-type cells (Kirchman *et al.*, 1993).

σ^G and σ^K

σ^G in the forespore directs the transcription of *spoIVB*, which encodes a 43 kDa protein that is required for the processing of pro-σ^K to σ^K (Lu *et al.*, 1990; Cutting *et al.*, 1991a). SpoIVB may play a role in the synthesis of the germ cell wall and this may stimulate processing. Consistent with this hypothesis, alleviating the requirement for pro-σ^K processing does not rescue sporulation in *spoIVB* mutant cells, indicating that SpoIVB plays some other role in sporulation in addition to its role in processing (Cutting *et al.*, 1991a). Alternatively, SpoIVB may directly interact with proteins involved in processing.

spoIVF is a two-cistron operon. Genetic studies indicate that one member of this operon, *spoIVFB*, encodes either the protease responsible for the processing of pro-σ^K to σ^K or a regulator of the processing enzyme (Cutting *et al.*, 1990; Cutting *et al.*, 1991b). The N-terminal portion of SpoIVFB resembles zinc proteases (S. Lu & L. Kroos, unpublished data).

SpoIVFB activity is regulated by proteins encoded by *spoIVFA*, the other member of the *spoIVF* operon, and *bofA* (bypass of forespore), since some mutations in these genes relieve the requirement for σ^G and *spoIVB* in the processing of pro-σ^K to σ^K (Cutting *et al.*, 1990). SpoIVFA has a positive and negative effect (Cutting *et al.*, 1991b). In its positive role, SpoIVFA appears to stabilize SpoIVFB activity, since a mutation in *spoIVFA* results in SpoIVFB becoming thermosensitive. In its negative role, SpoIVFA inhibits SpoIVFB until the σ^G-dependent signal is received from the forespore. BofA also has a negative effect until a signal is received from the forespore (Cutting *et al.*, 1990).

spoIVF and *bofA* are transcribed by σ^E RNA polymerase and their expression is thereby confined to the mother cell (Cutting *et al.*, 1991b; Ireton & Grossman, 1992a; Ricca *et al.*, 1992). The proteins encoded by these genes appear to have membrane spanning domains (Cutting *et al.*, 1991b; Ricca *et al.*, 1992). Based on these observations and the results from the genetic studies, it was proposed that these proteins form an oligomeric complex in the outer membrane of the forespore and govern pro-σ^K processing by sensing either a *spoIVB*-dependent morphological change in the membrane and/or a *spoIVB*-dependent signal from the forespore (Cutting *et al.*, 1991b; Ricca *et al.*, 1992).

Role of DNA-binding Proteins During Sporulation

The temporal pattern of gene expression appears to be established, in part, by DNA-binding proteins that act as both transcriptional activators and repressors.

Spo0A

As already discussed, the key event in the initiation of sporulation appears to be the phosphorylation of Spo0A *via* the phosphorelay system. Spo0A represses *abrB* transcription by σ^A RNA polymerase (Perego *et al.*, 1988). *abrB* encodes a transcriptional repressor, which blocks the transcription of several genes that are expressed during the transition from exponential growth to stationary phase. SpoOA stimulates the transcription of *spoIIA* (encoding σ^F) and *spoIIG* (encoding σ^E) by σ^H and σ^A RNA polymerase, respectively (Errington & Mandelstam, 1986; Savva & Mandelstam, 1986; Burbulys *et al.*, 1991; Satola *et al.*, 1991; Satola *et al.*, 1992). The repression of *abrB* occurs earlier than the stimulation of the *spoII* genes. Based on this observation, it was proposed that a low level of SpoOA results in the transcription of genes expressed during the transition from exponential growth to stationary phase and that the accumulation of SpoOA results in the transcription of *spoII* genes required for early stages of sporulation (Hoch, 1994).

SpoIIID

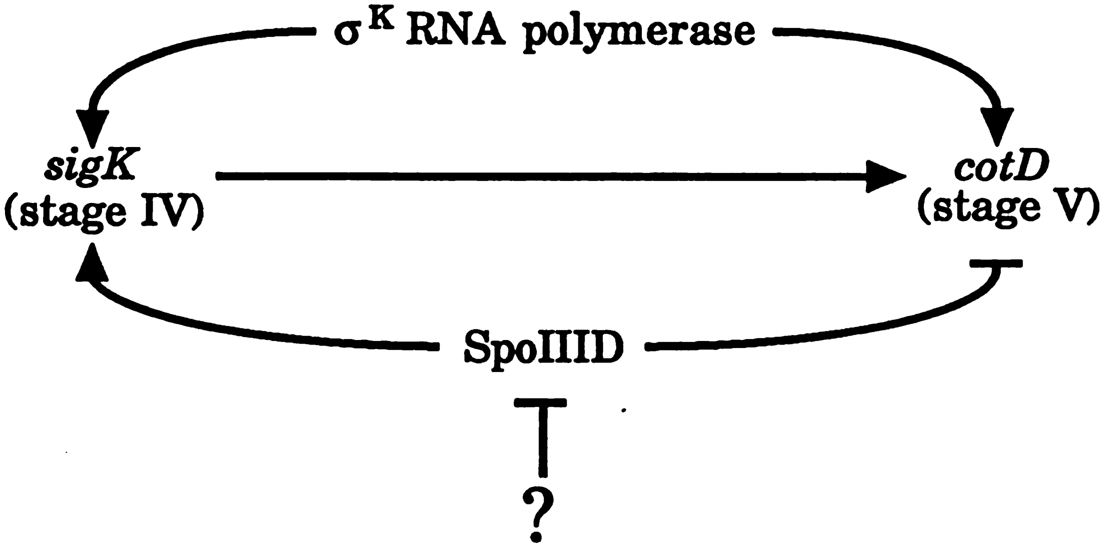
SpoIIID encodes a 10.8 kDa protein that contains a putative helix-turn-helix DNA-binding motif (Kroos *et al.*, 1989; Kunkel *et al.*, 1989; Stevens & Errington, 1990). Genetic studies and *in vitro* transcription experiments indicate that this protein stimulates and inhibits the transcription of σ^E- and σ^K-dependent genes (Table 1; Kunkel *et al.*, 1989; Stevens & Errington, 1990; Kroos *et al.*, 1989; Sato *et al.*, 1994; Ireton & Grossman, 1992a; Chapter II; J. Errington unpublished data; H. Ichikawa and L. Kroos unpublished data; B. Zhang and L. Kroos unpublished data). Since SpoIIID has both a positive and negative effect on

SpoIIID Effect	σ^E Regulon	σ^K Regulon
Stimulation	*spoIIID* ***sigK*** ***spoIVCA*** *spoVK*	*sigK*
Inhibition	*spoIIIA* ***bofA*** ***spoVD***	*cotC* *cotD* *cotX*

Table 1. Genes whose expression is affected by SpoIIID. Bold type indicates SpoIIID binds to the promoter and affects transcription *in vitro*. Plain type indicates SpoIIID affects the expression of ß-galactosidase from a fusion of the gene of interest to *lacZ*.

SpoIIID Effect	σ^E Regulon	σ^K Regulon
Stimulation	*spoIIID* ***sigK*** ***spoIVCA*** *spoVK*	***sigK***
Inhibition	*spoIIIA* ***bofA*** ***spoVD***	***cotC*** ***cotD*** ***cotX***

Figure 4. Temporal switch in the mother-cell pattern of gene expression. SpoIIID stimulates *sigK*, but represses *cotD* transcription by σ^K RNA polymerase. The inactivation and/or sequestering of SpoIIID (represented by a question mark) switches the mother-cell pattern of gene expression from *sigK* to *cotD* transcription.


σ^K RNA polymerase
sigK
(stage IV)
cotD
(stage V)
SpoIIID
?

transcription by σ^K RNA polymerase, it was postulated that the inactivation of SpoIIID establishes a switch in the mother-cell pattern of gene expression (Figure 4; Kroos & Losick, 1989).

GerE

gerE encodes an 8.5 kDa protein that contains a putative helix-turn-helix DNA-binding motif (Cutting & Mandelstam, 1986). This protein is similar to several response regulators (Kahn & Ditta, 1991). Genetic studies indicate that GerE stimulates or inhibits the expression of several spore coat genes by σ^K RNA polymerase. For example, in a *gerE* mutant background, *cotB* and *cotC* fail to be expressed, *cotD* is partially expressed, and *cotA* is overexpressed (Zheng *et al.*, 1992). Based on these observations, it was proposed that GerE may affect how coat proteins are deposited on the surface of the forespore and thereby affect the resistance properties of the mature spore (Zheng *et al.*, 1992). Consistent with this idea, *gerE* mutant spores are lysozyme-sensitive and germination defective (Feng & Aronson, 1986).

Significance

The regulatory features for controlling gene expression described above (i.e, sigma factors, anti-sigma factors, proteolytic processing, sequence-specific DNA-binding proteins, and coupling to morphogenesis) are present in other systems. A excellent example is flagellum biosynthesis in *Salmonella typhimurium*. The flagellar genes are grouped into 13 operons (Kutsukake *et al.*, 1988). These operons have been divided into three classes. Class I genes are required for the expression of class II genes and class II genes are required for the expression of class III genes (Kutsukake *et al.*, 1990). For example, *fliA* is a class II gene that encodes an alternative sigma factor, σ^F, which is required for the transcription of class III genes (Ohnishi *et al.*, 1990). Interestingly, *flgM* is another class II gene that encodes an anti-sigma factor, which regulates σ^F activity (Hughes *et al.*, 1993). The FlgM negative effect is relieved during flagellum biosynthesis by exporting it into the media (Hughes *et al.*, 1993). Export requires completion of part of the flagellum (Hughes *et al.*, 1993). This observation demonstrates the expression of class III genes is coupled to morphogenesis. Thus, studies on the model system of *B. subtilis* sporulation are likely to provide insight into the temporal and spatial regulation of gene expression in other organisms.

CHAPTER II

The SpoIIID Switch Protein Activates and Represses Transcription by Both Mother-Cell-Specific Forms of RNA Polymerase

"The rose and the thorn, and sorrow and gladness are linked together."

Saadi

Abstract

Mother-cell-specific gene expression during sporulation of *Bacillus subtilis* is controlled by σ^E and σ^K RNA polymerases. σ^E is required for the expression of genes during stage III (engulfment of the forespore), while σ^K is required for the expression of genes during stage IV (formation of the spore cortex) and stage V (formation of the spore coat). Previous studies indicated that SpoIIID could influence transcription by σ^K RNA polymerase *in vitro*. We demonstrate here that SpoIIID is a DNA-binding protein that recognizes specific sequences in the promoter regions and open reading frames of both σ^E- and σ^K-dependent genes. We also show that SpoIIID binding can activate or repress transcription by both forms of RNA polymerase. These results support the idea that the appearance and subsequent disappearance of SpoIIID plays a major role in establishing the mother-cell pattern of gene expression during stages III to V of sporulation.

Introduction

Under conditions of nutrient deprivation, the gram-positive bacterium *Bacillus subtilis* undergoes a series of morphological changes that culminate in the formation of an endospore (reviewed by Errington, 1993). The first easily observed morphological structure is an asymmetrically positioned septum that divides the bacterium into two compartments, the mother cell and the forespore. Both of these compartments receive a copy of the genome, but they realize alternative developmental fates because gene expression is regulated spatially. Spatial regulation is established by compartment-specific activation of sigma subunits of RNA polymerase (Losick & Stragier, 1992). Two mother-cell-specific sigma factors are σ^E (Driks & Losick, 1991) and σ^K (Kroos *et al.*, 1989; Stragier *et al.*, 1989). σ^E is required for the migration of the septum and engulfment of the forespore in a double membrane (stage III). σ^K is required for the deposition of cell-wall-like material called cortex between the membranes of the forespore (stage IV) (Cutting *et al.*, 1991a) and the synthesis of spore coat proteins that assemble on the surface of the forespore (stage V) (Kroos *et al.*, 1989; Zheng & Losick, 1990).

Gene expression in the mother cell is regulated temporally by the ordered appearance of σ^E, then σ^K. Temporal regulation in the mother cell also involves two transcription factors, SpoIIID and GerE, that affect gene expression driven by σ^E and/or σ^K RNA polymerase. Here we focus on transcriptional regulation by SpoIIID.

A mutation in *spoIIID*, which is predicted to encode a 10.8 kDa protein with a putative helix-turn-helix DNA-binding motif (Kunkel *et al.*, 1989; Stevens & Errington, 1990), affects the expression of several σ^E-dependent genes. For example, in *spoIIID* mutant cells *bofA* (encoding a protein that appears to inhibit processing of pro-σ^K to σ^K) is overexpressed (Ireton & Grossman, 1992a), but *spoIVCA* (encoding a putative recombinase that generates the composite *sigK* gene (Kunkel *et al.*, 1990; Sato *et al.*, 1990; Popham & Stragier, 1992)) and *sigK* (encoding pro-σ^K) fail to be expressed (Kunkel *et al.*, 1988; Sato *et al.*, 1994). However, it was unknown whether the SpoIIID protein affects σ^E-dependent transcription of these genes directly or indirectly.

SpoIIID was shown previously to stimulate *sigK* and inhibit *cotD* (encoding a spore coat protein) transcription by σ^K RNA polymerase *in vitro* (Kroos *et al.*, 1989). These effects appeared to result from SpoIIID binding to the DNA. Based on these observations, it was proposed that inactivation of SpoIIID establishes a switch in the mother-cell pattern of gene expression from *sigK* transcription at stage IV to *cotD* transcription at stage V (Kroos & Losick, 1989). Consistent with this model, the level of SpoIIID decreases sharply at the proper time during development (Halberg & Kroos, 1992).

Here we demonstrate that SpoIIID is a DNA-binding protein that recognizes specific sequences in the promoter regions and open reading frames (ORFs) of *bofA*, *spoIVCA*, *sigK*, and *cotD*. A consensus sequence for SpoIIID binding is proposed. We show that SpoIIID activates *spoIVCA* and *sigK* transcription, but represses *bofA* transcription by σ^E RNA polymerase *in vitro*. We also show that SpoIIID binding to the -35 region of *sigK* and *cotD* is sufficient to mediate the transcriptional effects that SpoIIID has on the transcription of these genes by σ^K RNA polymerase *in vitro*. These results suggest that the appearance and subsequent disappearance of SpoIIID plays a central role in determining the temporal pattern of mother-cell gene expression during the transition from stages III through V of sporulation.

Materials and Methods

DNase I footprinting

DNA fragments labelled at only one end were prepared as follows. For the analysis of *spoIVCA*, 10 μg of pUC118IVCP (Sato *et al.*, 1990) was digested with *Eco*RI and labelled using either the fill-in reaction of the Klenow fragment of DNA polymerase I and (α-^{32}P) dATP or by treating with alkaline phosphatase followed by phage T4 polynucleotide kinase and (γ-^{32}P) ATP. In both cases, the labelled DNA was digested with *Hind*III and the 654 bp *Eco*RI-*Hind*III fragment was purified after electrophoresis in a non-denaturing polyacrylamide gel using the crush and soak method described previously (Sambrook *et al.*, 1989), except double-stranded poly (dI·dC) (10 μg) was added to serve both as a carrier during the ethanol precipitation and as a competitor during the footprinting experiments. Similar end-labelling and recovery methods were used to prepare DNA fragments for analysis of *cotD*. A 279 bp *Eco*RI-*Nar*I fragment from pLRK100 (Kroos *et al.*, 1989) was labelled at the *Eco*RI site and a 443 bp *Eco*RI-*Hind*III fragment from pLRK100 was labelled at the *Hind*III site. For the analysis of *sigK*, 10 μg of pBK16 (Kroos *et al.*, 1989) was digested with *Xba*I and end-labelled as described above. The labelled DNA was digested with *Hind*III, which releases a 368 bp fragment containing a portion of *sigK* for footprinting, and *Eco*RI, which releases a fragment from the vector DNA that is sufficiently small (27 bp) so as not to interfere with footprinting. In this case, the vector DNA serves as competitor during footprinting reactions. Similarly, for the analysis of *bofA*, an approximately 400 bp *Eco*RI-*Bam*HI fragment from pIK132 (Ireton & Grossman, 1992a) was labelled at the *Eco*RI site for footprinting and *Xho*I was used to release a 33 bp labelled fragment from the vector DNA.

DNase I footprinting experiments were performed according to method 2 described by Zheng *et al.*, 1992, except 0.5 pmole of end-labelled DNA was used. SpoIIID was gel-purified from fractions of partially purified σ^K RNA polymerase as described previously (Kroos *et al.*, 1989).

DNA sequencing

End-labelled DNA fragments (described above) were subjected to the chemical cleavage reactions of Maxim and Gilbert as described previously (Maniatis *et al.*, 1982).

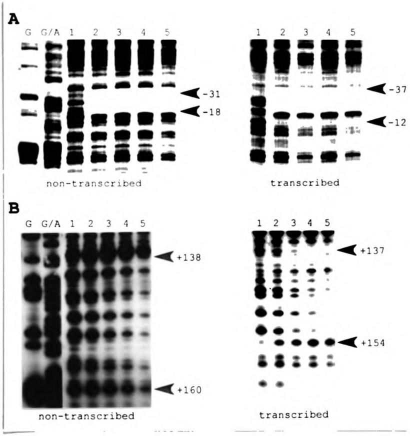
In vitro transcription

σ^E and σ^K RNA polymerases were partially purified from *sigK* (BK410; Kunkel *et al.*, 1989) and *gerE* (SC104; Kroos *et al.*, 1989) mutant cells, respectively, following the procedure for the partial purification of σ^K RNA polymerase described previously (Kroos *et al.*, 1989). The σ^K RNA polymerase was comparable in protein composition and in *cotD*- and *sigK*- transcribing activities to fraction 24 shown in Figure 2 of Kroos *et al.*, 1989. Transcription reactions (45 µl) were performed as described previously (Carter & Moran, 1986), except that RNA polymerase was allowed to bind to the DNA template for 10 minutes at 37°C before the addition of nucleotides. The labelled nucleotide was (α-^{32}P) CTP. Heparin (6 µg) was added 2 minutes after the addition of nucleotides to prevent reinitiation. After the reactions were stopped, 20 µl of each reaction mixture was subjected to electrophoresis and transcripts were detected by autoradiography. The signal intensities were quantitated using a Visage 110 Imager Analyzer (BioImage).

Results

SpoIIID binds to specific sequences in *spoIVCA*, *sigK*, *bofA*, and *cotD*. Gel-mobility shift assays indicated that SpoIIID binds to DNA fragments containing the *spoIVCA*, *sigK*, *bofA* or *cotD* promoter region (data not shown). To more precisely localize the binding of SpoIIID in these genes, DNase I protection experiments were performed. Radioactive DNA probes labelled separately on the non-transcribed or transcribed strand were incubated with SpoIIID and then mildly digested with DNase I. The resulting fragments were separated by gel electrophoresis and visualized by autoradiography. For the *spoIVCA* and *sigK* genes, sites in both the promoter region and ORF were protected by SpoIIID from DNase I digestion (Figure 1). In the case of *spoIVCA*, protection in the promoter region (site 1) spanned from -31 to -18 on the non-transcribed strand and from -37 to -12 on the transcribed strand (panel A), while protection in the ORF (site 2) spanned from +138 to +160 on the non-transcribed strand and from +137 to +154 on the transcribed strand (panel B). Extensive protection of site 1 was observed with 30 ng of SpoIIID, whereas only weak protection of site 2 was observed with 240 ng of SpoIIID, indicating that binding to site 1 is significantly stronger than binding to site 2. In the case of *sigK*, SpoIIID protected one site in the promoter region and two sites in

Figure 1. SpoIIID footprints on *spoIVCA* and *sigK*. Radioactive DNA probes separately end-labelled on the non-transcribed or transcribed strand were incubated in separate reactions with no protein (lane 1), 30 ng (lane 2), 60 ng (lane 3), 120 ng (lane 4), or 240 ng (lane 5) of gel-purified SpoIIID and then mildly digested with DNase I. The resulting DNA fragments were separated by electrophoresis on a 7% polyacrylamide gel containing 8 M urea alongside a sequencing ladder generated by chemical cleavage of one of the end-labelled DNA probes. (A) and (B) Footprints in the *spoIVCA* promoter region and ORF, respectively, identified using probes labelled at the *Eco*RI site located downstream of the transcriptional start site. (C), (D), and (E) Footprints in the *sigK* promoter region and ORF identified using probes labelled at the *Xba*I site located downstream of the transcriptional start site. Arrows indicate the boundaries of protection and numbers indicate positions relative to the transcriptional start site.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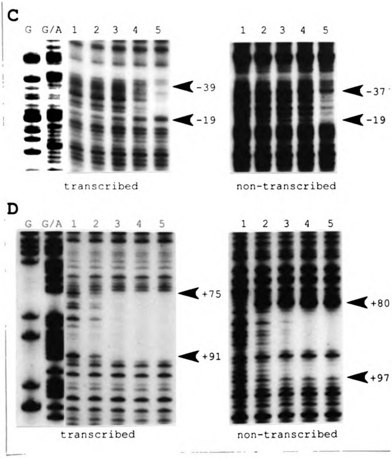
G
G/A
1
2
3
4
5
-31
-18
non-transcribed
1
2
3
4
5
-37
-12
transcribed
B
G
G/A
1
2
3
4
5
+138
+160
non-transcribed
1
2
3
4
5
+137
+154
transcribed


C
G
G/A
1
2
3
4
5
-39
-19
transcribed
1
2
3
4
5
-37
-19
non-transcribed
D
G
G/A
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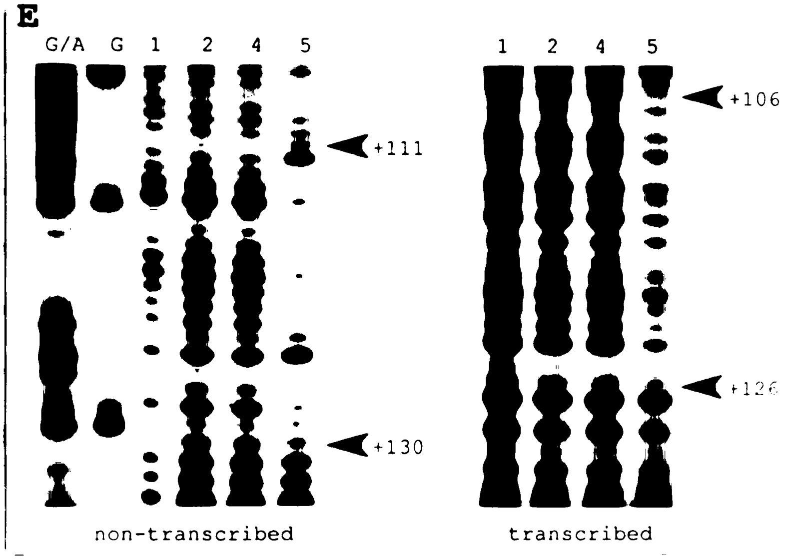
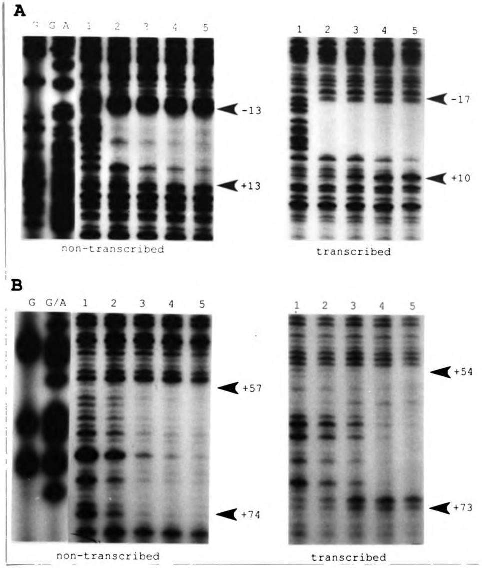
2
3
4
5
+75
+91
transcribed
1
2
3
4
5
+80
+97
non-transcribed

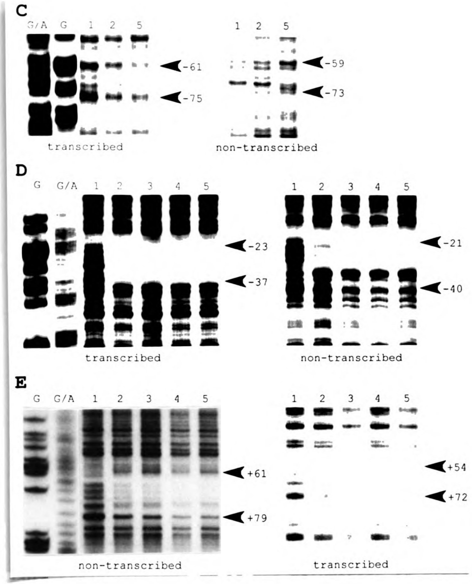

E
G/A
G
1
2
4
5
+111
+130
non-transcribed
1
2
4
5
+106
+126
transcribed

the ORF. Protection in the promoter region (site 1) spanned from -37 to -19 on the non-transcribed strand and from -39 to -19 on the transcribed strand (Figure 1C). Protected regions in the ORF (sites 2 and 3, respectively) spanned from +80 to +97 and +111 to +130 on the non-transcribed strand and from +75 to +91 and +106 to +126 on the transcribed strand (Figures 1D and 1E). Binding to site 2 was significantly stronger than binding to sites 1 and 3.

Similarly, for the *bofA* and *cotD* genes, sites in both the promoter region and ORF were protected by SpoIIID from DNase I digestion (Figure 2). In the case of *bofA*, protection in the promoter region (site 1) spanned from -13 to +13 on the non-transcribed strand and from -17 to +10 on the transcribed strand (panel A), while protection in the ORF (site 2) spanned from +57 to +74 on the non-transcribed strand and +54 to +73 on the transcribed strand (panel B). Binding to site 1 was slightly stronger than binding to site 2. In the case of *cotD*, SpoIIID protected two sites in the promoter region and one site in the ORF. Protected regions in the promoter region (sites 1 and 2, respectively) spanned from -73 to -59 and -40 to -21 on the non-transcribed strand and from -75 to -61 and -37 to -23 on the transcribed strand (Figures 2C and 2D). Protection in the ORF (site 3) spanned from +61 to +79 on the non-transcribed strand and from +54 to +72 on the transcribed strand (Figure 2E). Binding to sites 2 and 3 is comparable in strength and much stronger than

Figure 2. SpoIIID footprints on *bofA* and *cotD*. Radioactive DNA probes separately end-labelled on the non-transcribed or transcribed strand were incubated in separate reactions with no protein (lane 1), 30 ng (lane 2), 60 ng (lane 3), 120 ng (lane 4), or 240 ng (lane 5) of gel-purified SpoIIID and then mildly digested with DNase I. The resulting DNA fragments were separated by electrophoresis on a 7% polyacrylamide gel containing 8 M urea alongside a sequencing ladder generated by chemical cleavage of one of the end-labelled DNA probes. (A) and (B) Footprints in the *bofA* promoter region and ORF, respectively, identified using probes labelled at the *Eco*RI site located downstream of the transcriptional start site. (C) and (D) Footprints in the *cotD* promoter region identified using probes labelled at the *Eco*RI site located upstream of the transcriptional start site. (E) Footprints in the *cotD* ORF identified using probes labelled at the *Hind*III site located downstream of the transcriptional start site. Arrows indicate the boundaries of protection and numbers indicate positions relative to the transcriptional start site.


A
G G A 1 2 3 4 5
-13
+13
non-transcribed
1 2 3 4 5
-17
+10
transcribed
B
G G/A 1 2 3 4 5
+57
+74
non-transcribed
1 2 3 4 5
+54
+73
transcribed


C
G/A G 1 2 5
-61
-75
transcribed
1 2 5
-59
-73
non-transcribed
D
G G/A 1 2 3 4 5
-23
-37
transcribed
1 2 3 4 5
-21
-40
non-transcribed
E
G G/A 1 2 3 4 5
+61
+79
non-transcribed
1 2 3 4 5
+54
+72
transcribed

binding to site 1. The results of the DNase I protection experiments are summarized in Figure 3.

SpoIIID activates *spoIVCA* and *sigK* transcription, but represses *bofA* transcription by σE RNA polymerase *in vitro*. To determine how SpoIIID binding affects the transcription of *spoIVCA, sigK, and bofA,* linearized DNA templates were transcribed with partially purified σ^E RNA polymerase alone or in the presence of gel-purified SpoIIID (Figure 4). σ^E RNA polymerase produced run-off transcripts of the expected sizes from the *spoIVCA, sigK,* and *bofA* templates (panels A, B, and C, respectively). The presence of SpoIIID increased the *spoIVCA* (panel A, indicated by arrowheads, compare lanes 1 and 2 or lanes 3 and 4) and *sigK* (panel B, compare lanes 1 and 2) signals 5-fold and 6-fold, respectively, but it markedly reduced the *bofA* signal (panel C, compare lanes 1 and 2 or lanes 3 and 4). Thus, SpoIIID activates *spoIVCA* and *sigK* transcription, but it represses *bofA* transcription, by σ^E RNA polymerase *in vitro*. Activation of *spoIVCA* and *sigK* transcription and repression of *bofA* transcription by SpoIIID *in vitro* are consistent with the effects of a *spoIIID* mutation on the expression of *lacZ* fusions to these promoters *in vivo* (Kunkel *et al.*, 1988; Ireton & Grossman, 1992a; Sato *et al.*, 1994).

Surprisingly, σ^E RNA polymerase produced another run-

Figure 3. Position of SpoIIID binding sites in *spoIVCA*, *sigK*, *bofA* and *cotD*. Overlining and underlining indicate regions on the non-transcribed and transcribed strands, respectively, protected by SpoIIID from DNase I digestion (Figures 1 and 2). The dashed portion of a line indicates a region of uncertain protection due to a lack of DNase I digestion in this region. Asterisks indicate positions of enhanced cleavage by DNase I upon SpoIIID binding. Numbers refer to positions relative to the transcriptional start site. Nucleotide sequences upstream of the transcriptional start sites of *spoIVCA*, *sigK*, and *bofA* are aligned with respect to conserved nucleotides in the -10 and -35 regions of promoters transcribed by σ^E RNA polymerase, shown at the top of the diagram (Roels *et al.*, 1992). k means G or T and m means A or C. Nucleotide sequences upstream of the transcriptional start sites of *sigK* and *cotD* are aligned with respect to conserved nucleotides in the -10 and -35 regions of promoter transcribed by σ^K RNA polymerase, shown above the *sigK* and *cotD* sequences (Zheng *et al.*, 1992). Matches to the consensus sequences are shown as bold, capital letters.

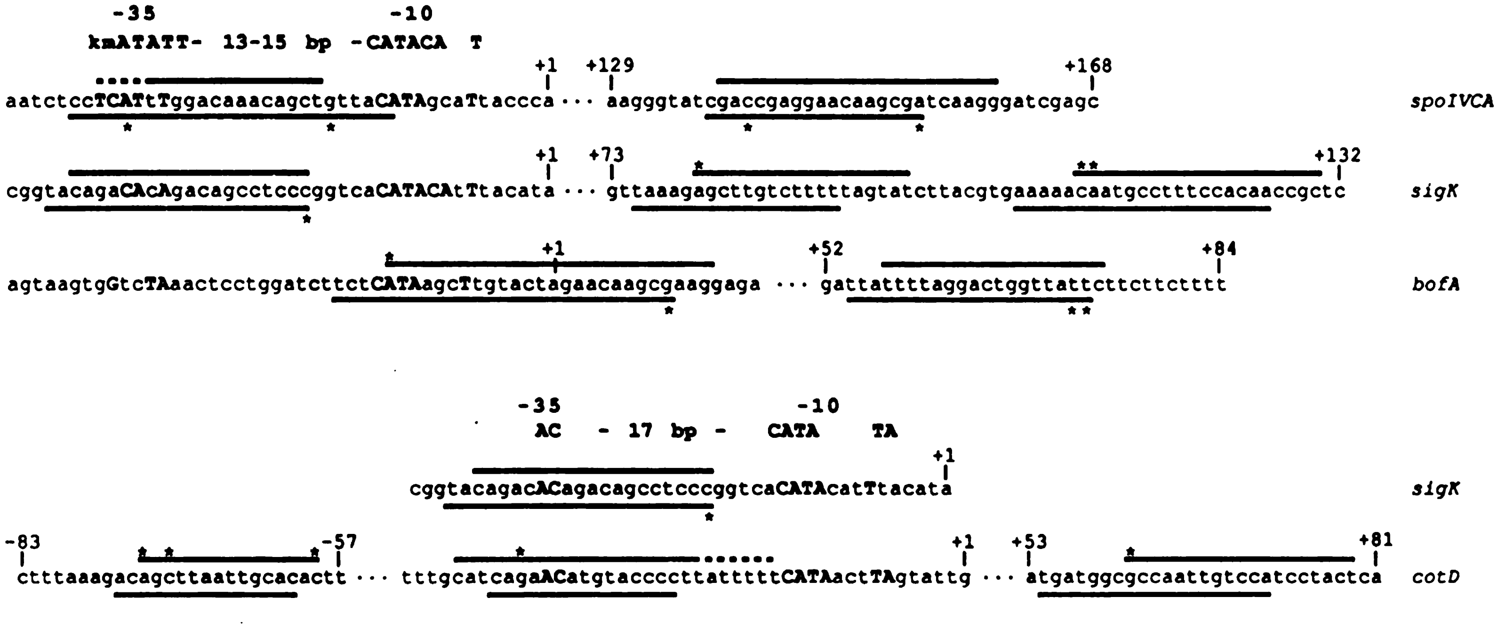
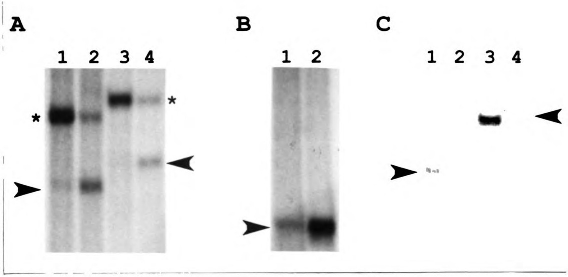

-35
-10
kmATATT- 13-15 bp -CATACA T
+1
+129
+168
aatctccTCATtTggacaaacagctgttaCATAgcaTtaccca ··· aagggtatcgaccgaggaacaagcgatcaagggatcgagc
spoIVCA
+1
+73
+132
cggtacagaCAcAgacagcctcccggtcaCATACAtTtacata ··· gttaaagagcttgtctttttagtatcttacgtgaaaaacaatgcctttccacaaccgctc
sigK
+1
+52
+84
agtaagtgGtcTAaactcctggatcttctCATAagcTtgtactagaacaagcgaaggaga ··· gattattttaggactggttattcttcttctttt
bofA
-35
-10
AC - 17 bp - CATA TA
+1
cggtacagacACagacagcctcccggtcaCATAcatTtacata
sigK
-83
-57
+1
+53
+81
ctttaaagacagcttaattgcacactt ··· tttgcatcagaACatgtaccccttatttttCATAactTAgtattg ··· atgatggcgccaattgtccatcctactca
cotD

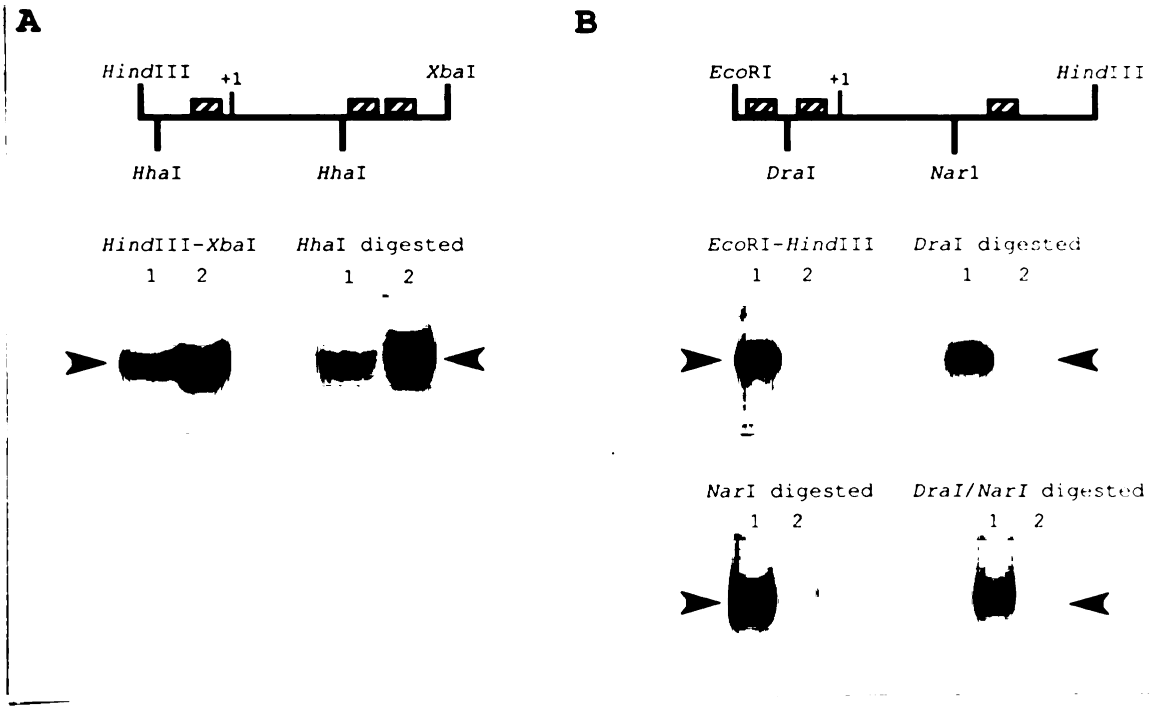
Figure 4. Effects of SpoIIID on *spoIVCA*, *sigK*, and *bofA* transcription *in vitro*. Linearized plasmid DNA (1 μg) was transcribed with partially purified σ^E RNA polymerase (200 ng) alone or in the presence of gel-purified SpoIIID (120 ng). Run-off transcripts were electrophoresed in a 5% polyacrylamide gel containing 8 M urea. Arrowheads denote the positions of run-off transcripts of the expected sizes, while asterisks denote the positions of run-off transcripts of unexpected sizes. The sizes of run-off transcripts were estimated from the migration of end-labelled fragments of *Msp*I-digested pBR322. (A) *spoIVCA* transcription from pUC118IVCP digested with *Kpn*I (lanes 1 and 2, 193-base transcript) or *Eco*RI (lanes 3 and 4, 201-base transcript) with σ^E RNA polymerase alone (lanes 1 and 3) or in the presence of SpoIIID (lanes 2 and 4). (B) *sigK* transcription from pBK16 digested with *Xba*I (170-base transcript) with σ^E RNA polymerase alone (lane 1) or in the presence of SpoIIID (lane 2). (C) *bofA* transcription from pIK132 digested with *Eco*RI (lanes 1 and 2, 134-base transcript) or *Xba*I (lanes 3 and 4, 164-base transcript) with σ^E RNA polymerase alone (lanes 1 and 3) or in the presence of SpoIIID (lanes 2 and 4).


A
1
2
3
4
*
*
B
1
2
C
1
2
3
4

off transcript which is approximately 40 bases longer than the expected product from the *spoIVCA* template (Figure 4A, indicated by asterisks). The longer transcript appears to originate from a site upstream of the *spoIVCA* transcriptional start site since its size varies in the same manner as the size of the *spoIVCA* transcript when templates are cleaved at different downstream restriction sites. The presence of SpoIIID markedly reduced the longer transcript signal (Figure 4A, compare lanes 1 and 2 or lanes 3 and 4). A possible explanation for these results is presented in the Discussion.

SpoIIID binding in the -35 region is sufficient to activate *sigK* transcription and repress *cotD* transcription by σK RNA polymerase *in vitro*. SpoIIID was shown previously to stimulate *sigK* transcription and inhibit *cotD* transcription by σ^K RNA polymerase *in vitro* (Kroos *et al.*, 1989). To determine which SpoIIID binding sites are required for these effects, *sigK* and *cotD* templates containing different combinations of SpoIIID binding sites were transcribed with partially purified σ^K RNA polymerase alone or in the presence of gel-purified SpoIIID (Figure 5). The presence of SpoIIID increased *sigK* transcription 3-fold from a *Hind*III-*Xba*I fragment containing sites 1-3, and 3-fold from this template after it was digested with *Hha*I (panel A). The presence of SpoIIID also increased transcription to a

Figure 5. Effects of SpoIIID on the *in vitro* transcription of *sigK* and *cotD* templates containing different combinations of SpoIIID binding sites. Isolated fragments containing a portion of *sigK* or *cotD* served as templates directly, or were digested with restriction enzymes prior to serving as templates. The fragments (300 ng total DNA in each case) were transcribed separately with partially purified σ^K RNA polymerase (200 ng) alone or in the presence of gel-purified SpoIIID (120 ng). Run-off transcripts were electrophoresed in a 5% polyacrylamide gel containing 8 M urea. Arrowheads denote the positions of run-off transcripts of the expected sizes in each panel, as judged from the migration of end-labelled fragments of MspI-digested pBR322. (A) For *sigK,* a *Hind*III-*Xba*I fragment isolated from pBK16, and this fragment digested with *Hha*I, were transcribed with σ^K RNA polymerase alone (lane 1) or in the presence of SpoIIID (lane 2). The expected sizes of the run-off transcripts from the *Hind*III-*Xba*I fragment and the *Hha*I subfragment are 170 and 60 bases, respectively. (B) For *cotD,* an *Eco*RI-*Hind*III fragment isolated from pLRK100, and this fragment digested with *Dra*I, *Nar*I, or both *Dra*I and *Nar*I, were transcribed with σ^K RNA polymerase alone (lane 1) or in the presence of SpoIIID (lane 2). The expected size of the run-off transcript from the *Eco*RI-*Hind*III fragment or this fragment digested with *Dra*I is 225 bases. The expected size of the run-off transcript from the *Eco*RI-*Hind*III fragment digested with *Nar*I or with *Dra*I and *Nar*I is 54 bases.


A
HindIII
+1
XbaI
HhaI
HhaI
HindIII-XbaI
1 2
HhaI digested
1 2
B
EcoRI
+1
HindIII
DraI
NarI
EcoRI-HindIII
1 2
DraI digested
1 2
NarI digested
1 2
DraI/NarI digested
1 2

similar degree from the isolated *Hha*I fragment (data not shown). Thus, SpoIIID binding in the -35 region of *sigK* is sufficient to activate transcription by σ^K RNA polymerase *in vitro*. The presence of SpoIIID markedly reduced *cotD* transcription from an *Eco*RI-*Hind*III template containing sites 1-3, and from this template after it was digested with *Dra*I, *Nar*I, or both *Dra*I and *Nar*I (panel B). The presence of SpoIIID also markedly reduced transcription from the isolated *Dra*I-*Hind*III and *Eco*RI-*Nar*I fragments (data not shown). Thus, SpoIIID binding in the -35 region of *cotD* is sufficient to repress transcription by σ^K RNA polymerase *in vitro*.

Discussion

We have shown that SpoIIID is a DNA-binding protein that positively and negatively affects transcription by both mother-cell-specific forms of RNA polymerase. SpoIIID activates *spoIVCA* and *sigK* transcription, but represses *bofA* transcription, by σ^E RNA polymerase *in vitro*. SpoIIID also activates *sigK* transcription, but represses *cotD* transcription, by σ^K RNA polymerase *in vitro*. These results are consistent with the effects of a *spoIIID* mutation on gene expression *in vivo* (Kunkel *et al.*, 1988; Zheng & Losick, 1990; Ireton & Grossman, 1992a; Sato *et al.*, 1994). Taken together, these observations suggest that SpoIIID plays a direct role in establishing the temporal pattern of mother-cell gene expression by both activating and repressing transcription directed by two forms of RNA polymerase that appear sequentially during sporulation.

The -10 and -35 regions of the promoters used in this study are aligned with the proposed consensus sequences for σ^E and σ^K-dependent promoters in Figure 3. The *spoIVCA, sigK,* and *bofA* promoters all show considerable similarity to the consensus for σ^E-dependent promoters. It is not obvious from this sequence comparison why, in the absence of SpoIIID, the *bofA* promoter is more highly transcribed by σ^E RNA polymerase *in vitro* than the *spoIVCA* and *sigK* promoters (Figure 4). Similarly, both the *sigK* and *cotD* promoters match the consensus sequence for σ^K-dependent promoters quite well, yet the *cotD* promoter is more highly transcribed by σ^K RNA polymerase *in vitro* unless SpoIIID is added (Figure 5).

Comparison of sequences in the SpoIIID binding sites in the promoter regions and open reading frames of *spoIVCA*, *sigK*, *bofA*, and *cotD* reveals an apparent consensus for SpoIIID binding, WWRRACAR-Y (W=A or T, R=purine, and Y=pyrimidine; Figure 6A). Interestingly, the ACA sequence in this consensus is similar to the ATA and AC sequences found in the -35 region of strong σ^E- and σ^K-dependent promoters, respectively (Figure 3). This may reflect similarity between the putative helix-turn-helix DNA-binding motif of SpoIIID (Kunkel *et al.*, 1989; Stevens & Errington, 1990) and regions 4.2 of σ^E and σ^K, which are predicted to adopt helix-turn-helix structures that interact with the -35 region of cognate promoters (Helmann & Chamberlin, 1988; Lonetto *et al.*, 1992). For example, all three proteins have a serine residue at the same position in their putative recognition helix. This serine may hydrogen bond to adenine in the consensus sequences.

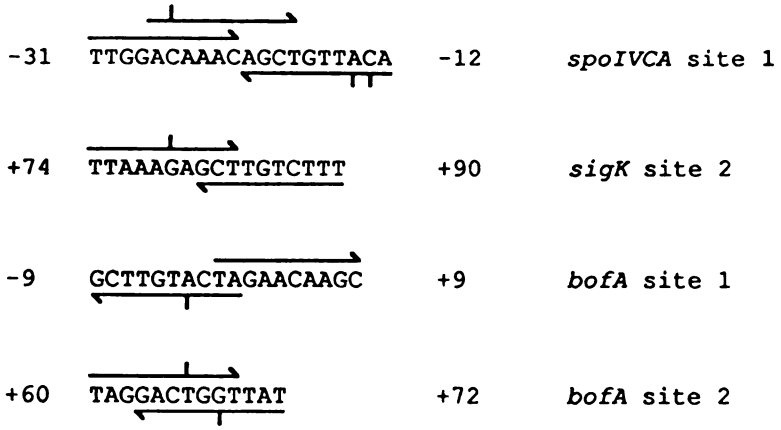
Some proteins containing a helix-turn-helix DNA-binding motif are dimeric (e.g., catabolite activator protein and tryptophan repressor) and bind to sequences that exhibit dyad

Figure 6. Alignment of sequences within SpoIIID binding sites and the arrangement of additional matches to the proposed consensus sequence for SpoIIID binding. (A) Nucleotide sequences protected from DNase I by SpoIIID are aligned with a proposed consensus sequence, which is shown at the bottom. Nucleotides that match this consensus are shown as bold, capital letters. R means purine, Y means pyrimidine, and W means A or T. Note: The sequences shown for *sigK* site 2 and *cotD* site 3 are from the opposite DNA strand of that shown in Figure 3. (B and C) Arrangement of matches to the SpoIIID consensus in strong and weak SpoIIID binding sites, respectively. Arrows point 5' to 3' and indicate sequences matching the SpoIIID consensus, with tick marks indicating mismatches. Note: There is a third 7 out of 9 nucleotide match to the SpoIIID consensus to *cotD* site 1, but it is not shown because it contains two mismatches within the highly conserved ACA sequence.

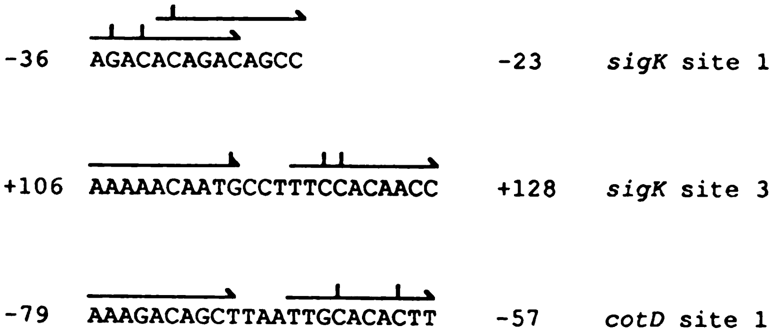
A

-31	**TTGGACAAaC**	-22	*spoIVCA* site 1
+143	**AgGAACAAgC**	+152	*spoIVCA* site 2
-32	**AcAGACAGcC**	-23	*sigK* site 1
+90	**AAAGACAAgC**	+81	*sigK* site 2
+106	**AAAAACAAtg**	+115	*sigK* site 3
-1	**TAGAACAAgC**	+9	*bofA* site 1
+60	**TAGGACtGgT**	+69	*bofA* site 2
-79	**AAAGACAGcT**	-70	*cotD* site 1
-37	**cAGAACAtgT**	-28	*cotD* site 2
+73	**ATGGACAAtT**	+64	*cotD* site 3
	WWRRACAR-Y		Consensus

B



C



symmetry (Otwinowski *et al.*, 1988; Schultz *et al.*, 1991). Each sequence of the dyad is bound by one of the helix-turn-helix motifs present in the dimer. The consensus sequence we propose for SpoIIID binding does not exhibit dyad symmetry. Consistent with this observation, SpoIIID purified from sporulating *B. subtilis* is primarily in a monomeric state (B. Zhang and L. Kroos, unpublished data). SpoIIID probably binds to the proposed consensus as a monomer. However, some of the sites bound most strongly by SpoIIID exhibit a second good match (i.e., at least 7 out of 9) to the consensus in inverted orientation relative to the best match (Figure 6B), suggesting possible cooperative interactions between monomers. Some of the weakly bound sites show a second good match in direct orientation (Figure 6C). Mutational studies, and biochemical experiments to determine the number of SpoIIID monomers bound at particular sites, will be required to determine the significance of various arrangements of sequences matching the proposed consensus.

Why does SpoIIID bind to both the promoter region and open reading frame of all four genes tested? On the basis of chance alone, a perfect match to the proposed consensus should occur once in about 4 kb of random sequence. In the case of *sigK*, three SpoIIID binding sites were found within a 170 bp region. However, binding of SpoIIID to site 1 in the promoter region is sufficient to stimulate transcription, as evidenced by our *in vitro* result (Figure 5A) and the *spoIIID* dependence *in vivo* of a fusion between the *sigK* promoter region (-106 to +4) and the *E. coli lacZ* gene (Kunkel *et al.*, 1988). These results do not rule out the possibility that SpoIIID binding sites 2 and 3 in the *sigK* ORF may help activate transcription *in vivo*. AlgR1, a response regulator protein from *Pseudomonas aeruginosa*, stimulates *algC* promoter activity by binding to three sites located at -94 to -81, +161 to +174 (in the leader sequence), and +389 to +403 (in the structural gene) (Fujiwara *et al.*, 1993). However, binding to the upstream site alone is sufficient to mediate 14% of the stimulatory effect *in vivo* (Fujiwara *et al.*, 1993).

SpoIIID binds to three sites within a 160 bp region encompassing the *cotD* transcriptional start site, but binding to site 2 in the promoter region is sufficient to repress transcription *in vitro* (Figure 5B). It remains possible that SpoIIID binding to sites 1 and/or 3 facilitates repression. This could involve interactions between bound SpoIIID molecules and looping of intervening DNA, as appears to be important for repression of the *gal* and *ara* operons in *E. coli* (Martin *et al.*, 1986; Mandal *et al.*, 1990). SpoIIID binding to DNA enhanced cleavage by DNase I at one or both ends of each protected region (Figures 1 to 3), perhaps indicating that SpoIIID bends the DNA upon binding.

Why does SpoIIID binding in the -35 region of *sigK* and *cotD* have an opposite effect on transcription *in vitro*? In the consensus sequence for SpoIIID binding, there is an absolutely conserved ACA sequence (Figure 6A). The C of this trinucleotide is at position -27 in the *sigK* promoter and at position -32 in the *cotD* promoter (Figure 3). Thus, SpoIIID may bind to opposite faces of the DNA in the -35 region of the *sigK* and *cotD* promoters. If so, the interactions between SpoIIID and σ^K RNA polymerase would be expected to be different, perhaps accounting for the opposite effects on *sigK* and *cotD* promoter activity. In particular, we propose that repression of *cotD* transcription may result from direct competition between SpoIIID and σ^K RNA polymerase for contacts with the AC sequence at positions -33 and -32 (Figure 3). Activation of *sigK* transcription may result from interactions between SpoIIID and either σ^K or the α subunits of RNA polymerase, based on analogy with emerging evidence for several prokaryotic transcriptional activators (Ishihama, 1993).

SpoIIID activates *spoIVCA* transcription, but represses *bofA* transcription by σ^E RNA polymerase (Figure 4). In the case of *spoIVCA,* a perfect match to the consensus sequence for SpoIIID binding is present at nearly the identical position (one nucleotide closer to the transcriptional start site) as in the *sigK* promoter. This observation is consistent with the idea that SpoIIID activates transcription by binding to a particular face of the DNA helix. Moreover, it suggests that SpoIIID activates transcription by σ^E and σ^K

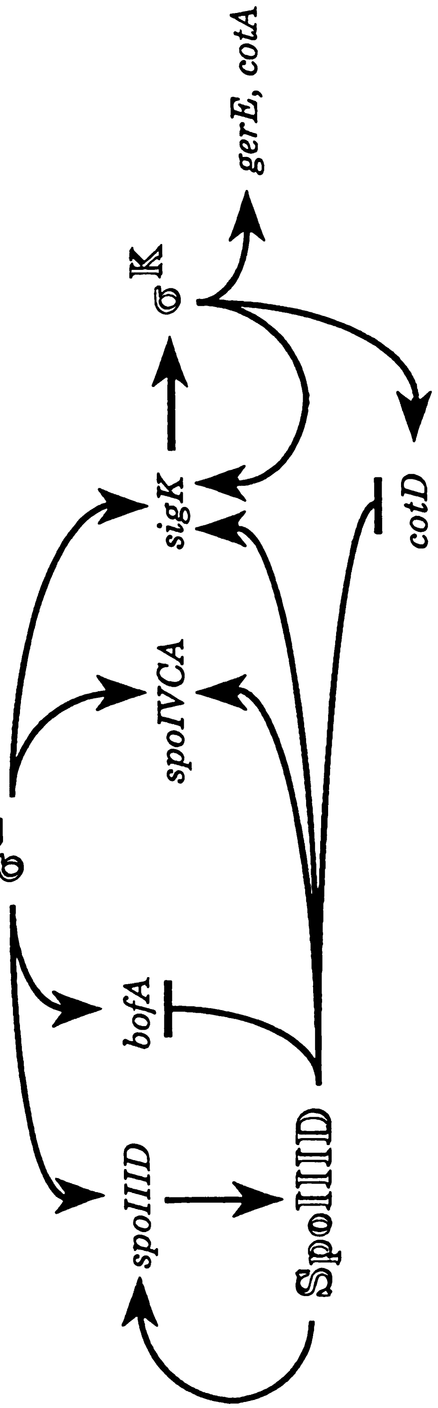
RNA polymerase *via* a similar mechanism. However, the possible role of SpoIIID binding to site 2 in the *spoIVCA* ORF has not been explored since site 2 was present in all of our *in vitro* experiments, as well as in the reported *in vivo* experiments with a *spoIVCA-lacZ* fusion (Sato *et al.*, 1994). In the case of *bofA*, SpoIIID binding site 1 is centered at the transcriptional start site. This region is exclusively occupied by repressors when considering *E. coli* transcriptional regulators, because a protein bound here probably either prevents RNA polymerase from recognizing the promoter or prevents a later step in transcriptional initiation (Collado-Vides *et al.*, 1991). Interestingly, other proteins that function as both an activator and repressor (e.g., fumarate and nitrate regulatory protein) activate transcription by binding in the -35 region of a promoter and repress transcription by binding near the transcriptional start site (Eiglmeier *et al.*, 1989). Binding of SpoIIID to site 2 in the *bofA* ORF may be required for repression. Both sites 1 and 2 were present in our *in vitro* experiments and in a *bofA-lacZ* fusion that showed seven-fold higher expression in *spoIIID* mutant cells than in wild-type cells.

σ^E RNA polymerase produced an unexpected transcript from the *spoIVCA* template *in vitro* (Figure 4A). This transcript appears to originate approximately 40 bp upstream of the *in vivo spoIVCA* transcriptional start site. Inspection of this region reveals a 7 bp sequence (TCATTGA) at positions -78 to

-72 and an 8 bp sequence (TATAGTTA) at positions -57 to -50, which resemble the -35 and -10 regions of σ^E-dependent promoters, respectively. This promoter does not appear to be active *in vivo,* based on both S1 nuclease protection and primer extension experiments (Sato *et al.*, 1990; Sato *et al.*, 1994). Interestingly, transcription that appears to be initiated from this promoter is repressed by SpoIIID *in vitro* (Figure 4A). The observed repression may result from SpoIIID binding to site 1, which lies just downstream of the approximate transcriptional start site of the *in vitro* promoter.

SpoIIID plays a critical role in establishing the mother-cell pattern of gene expression by affecting transcription by σ^E and σ^K RNA polymerase (Figure 7). σ^E RNA polymerase transcribes *spoIIID* (Tatti *et al.*, 1991). As SpoIIID accumulates, it affects the transcription of other σ^E-dependent genes. For example, SpoIIID represses *bofA* transcription (Figure 4; (Ireton & Grossman, 1992a)), but activates the transcription of its own gene (Kunkel *et al.*, 1990; Stevens & Errington, 1990) as well as transcription of *spoIVCA* and *sigK* (Figure 4; (Kunkel *et al.*, 1988; Sato *et al.*, 1994)). Other σ^E-dependent genes repressed by SpoIIID may include *spoIIIA* and *spoVD,* since (1) *spoIIIA*- and *spoVD*-directed ß-galactosidase activity are significantly

Figure 7. Regulatory effects of σ^E, SpoIIID, and σ^K on mother-cell gene expression. Arrows represent activation, while bars represent repression.


spoIIID
SpoIIID
bofA
σE
spoIVCA
sigK
σK
cotD
gerE, cotA

higher in *spoIIID* mutant cells than in wild-type cells (Illing & Errington, 1991b; Daniel *et al.*, 1994), and (2) SpoIIID represses *spoVD* transcription by σ^E RNA polymerase *in vitro* (B. Zhang and L. Kroos, unpublished data). Other σ^E-dependent genes activated by SpoIIID may include *cotE* and *spoVK*, since *cotE*- and *spoVK*-directed ß-galactosidase activity are markedly reduced in *spoIIID* mutant cells (Zheng *et al.*, 1988; Errington *et al.*, 1989). Thus, an increase in the level of SpoIIID switches the mother-cell pattern of gene expression, repressing the transcription of some σ^E-dependent genes and activating transcription of others.

The primary translation product of *sigK* is pro-σ^K, a transcriptionally inactive precursor protein (Kroos *et al.*, 1989; Stragier *et al.*, 1989). pro-σ^K is proteolytically processed to active σ^K by removing amino acids from its N-terminus (Lu *et al.*, 1990). As σ^K becomes available, it transcribes its own gene (Kunkel *et al.*, 1988; Kroos *et al.*, 1989) as well as σ^K-dependent genes whose expression is unaffected by SpoIIID (e.g., *cotA* and *gerE*; R. Halberg and L. Kroos, unpublished data). Accumulation of σ^K causes a decrease in the level of SpoIIID, allowing *cotD* transcription to begin (Halberg & Kroos, 1992). Thus, a decrease in the level of SpoIIID appears to switch the mother-cell pattern of gene expression from σ^K-dependent genes like *sigK*, whose expression is activated by SpoIIID, to σ^K-dependent genes like *cotD*, whose expression is repressed by SpoIIID. Transcription of two other σ^K-dependent genes, *cotC* and *cotX*, is repressed by SpoIIID *in vitro* (H. Ichikawa and L. Kroos, unpublished data). GerE is a DNA-binding protein that reinforces the switch brought about by the disappearance of SpoIIID, since its level rises as σ^K accumulates and its effects on *sigK*, *cotD*, *cotC*, and *cotX* transcription are opposite the effects of SpoIIID (Zheng *et al.*, 1992; Zhang *et al.*, 1994). In addition, GerE activates the transcription of several other *cot* genes and represses *cotA* transcription (Zheng *et al.*, 1992; Zhang *et al.*, 1994). GerE appears to be the principal regulator of σ^K-dependent gene expression, while SpoIIID may "fine-tune" the expression of particular *cot* genes by affecting the time and/or level of expression, possibly enhancing spore coat assembly. The results presented here demonstrate that SpoIIID is also a direct regulator of σ^E-dependent gene transcription, including transcription of *spoIVCA* and *sigK*, which is essential for production of σ^K.

CHAPTER III

Fate of the SpoIIID Switch Protein During Bacillus subtilis Sporulation Depends on the Mother-Cell Sigma Factor, σ^K

"To be or not to be..."

William Shakespeare

J. Mol. Biol. (1992) **228**, 840–849

Fate of the SpoIIID Switch Protein during *Bacillus subtilis* Sporulation Depends on the Mother-cell Sigma Factor, σ^K

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Sporulation of *Bacillus subtilis* involves the differentiation of two cell types, the mother cell and the forespore. Two key regulators of mother-cell gene expression are SpoIIID, a DNA-binding protein that activates or represses transcription of many different genes, and σ^K, a subunit of RNA polymerase that directs the enzyme to transcribe genes encoding proteins that form the spore coat. Previous studies showed that SpoIIID is needed to produce σ^K, but suggested that SpoIIID represses σ^K-directed transcription of genes encoding spore coat proteins. Here we show that a feedback loop connects the levels of σ^K and SpoIIID, such that production of σ^K leads to a decrease in the level of SpoIIID. The existence of the feedback loop was demonstrated by using antibodies prepared against SpoIIID to measure the level of SpoIIID during sporulation of wild-type cells, mutants defective in σ^K production, and a mutant engineered to produce σ^K earlier than normal. The feedback loop operates at the level of synthesis and/or stability of *spoIIID* mRNA, as demonstrated by measuring the level of *spoIIID* mRNA during sporulation of wild-type cells and mutants defective in σ^K production. Our results suggest that a rise in the level of σ^K during the stage (IV) of spore cortex formation causes a decrease in the level of SpoIIID, which, at least in part, establishes the switch to the stage V (spore coat formation) pattern of mother-cell gene expression.

Keywords: transcription factor; *Bacillus subtilis*; sporulation; σ factor; feedback loop

1. Introduction

In response to starvation, the Gram-positive bacterium *Bacillus subtilis* undergoes a series of morphological changes that result in the formation of an endospore (Smith *et al.*, 1989). The first easily observed morphological structure that is specific to the sporulation process is an asymmetrically positioned septum, which divides the bacterium into mother-cell and forespore compartments. Both of these compartments receive a copy of the genome, but differential gene expression in the two compartments drives further morphological change including migration of the septum and engulfment of the forespore in a double membrane (stage III), disposition of cell-wall-like material called cortex between the membranes surrounding the forespore (stage IV), and synthesis in the mother cell of spore coat proteins that assemble on the surface of the forespore (stage V). The developmental process culminates with the lysis of the mother cell to release the endospore.

Gene expression in the mother-cell compartment during stages III to V of sporulation is controlled, in part, by two regulatory proteins, SpoIIID and σ^K. The gene encoding SpoIIID (*spoIIID*) is transcribed predominantly, if not exclusively, in the mother cell by RNA polymerase containing σ^E (Kunkel *et al.*, 1989; Stevens & Errington, 1990; Tatti *et al.*, 1991), which appears to be active only in the mother cell (Driks & Losick, 1991). SpoIIID is a 10·8 kDa DNA-binding protein that activities or represses transcription of many different genes (Kroos *et al.*, 1989; our unpublished results). σ^K is a sigma subunit of RNA polymerase that directs the enzyme to transcribe genes whose products are needed for formation of the spore cortex and coat during morphological stages IV and V, respectively (Kroos *et al.*, 1989; Zheng *et al.*, 1992).

SpoIIID regulates production of σ^K by at least two mechanisms. First, SpoIIID is required for the chromosomal DNA rearrangement that generates the composite gene (*sigK*) encoding σ^K (Stragier *et al.*, 1989; Kunkel *et al.*, 1990). Second, SpoIIID is required for the transcription of *sigK* (Kunkel *et al.*, 1988), which appears to be driven initially by σ^E RNA polymerase (our unpublished results), then by σ^K RNA polymerase (Kroos *et al.*, 1989). Another

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mechanism controlling σ^K production is a proteolytic processing event that generates active σ^K from the primary translation product of *sigK*, called pro-σ^K, and several lines of evidence support the view that pro-σ^K processing couples mother-cell gene expression to events occurring in the forespore (Cutting *et al.*, 1990, 1991*a*,*b*; Lu *et al.*, 1990).

In addition to its role in activating transcription of *sigK*, SpoIIID markedly represses transcription of *cotD* (encoding a spore coat protein; Donovan *et al.*, 1987) by σ^K RNA polymerase *in vitro* (Kroos *et al.*, 1989). Here we demonstrate that the level of SpoIIID decreases at the appropriate time during sporulation to produce a switch from *sigK* to *cotD* expression. We also present evidence that the SpoIIID decrease is controlled by the production of active σ^K *via* a negative feedback loop acting on the synthesis and/or stability of *spoIIID* mRNA. These results suggest that a rise in the level of σ^K and a concomitant decrease in the level of SpoIIID govern the transition from the stage IV to the stage V pattern of mother-cell gene expression.

2. Materials and Methods

(a) *Bacterial strains*

B. subtilis strains were provided by R. Losick except for BRH3, which was generated by transforming competent 522.2 (*gerE36*, *trpc2*; Errington & Mandelstam, 1986) prepared as described previously (Dubnau & Davidoff-Abelson, 1971) with chromosomal DNA isolated from VO48 (*spoIVCBΔ19*; Cutting *et al.*, 1990) and selecting chloramphenicol-resistant colonies. All strains are isogenic with the Spo$^+$ strain PY79 (Youngman *et al.*, 1984) except for 522.2 and BRH3, which are isogenic with the Spo$^+$ strain SG38 (Errington & Mandelstam, 1986).Use of the specialized transducing phage SP*β*::*cotD-lacZ* (obtained from L. Zheng and R. Losick) has been described (Zheng & Losick, 1990).

(b) *Growth and sporulation*

Sporulation was induced by resuspending growing cells in SM medium as described previously (Sterlini & Mandelstam, 1969). The onset of sporulation (T_0) is defined as the time of resuspension.

(c) *Preparation of antibodies*

SpoIIID was partially purified from sporulating *B. subtilis* as described previously (Kroos *et al.*, 1989). Proteins in DNA-cellulose column fractions were precipitated in 10% trichloroacetic acid, resuspended and boiled for 5 min in sample buffer (0·125 M-Tris·HCl, pH 6·8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0·1% (w/v) bromophenol blue), and subjected to SDS/PAGE (18% polyacrylamide gel; Thomas & Kornberg, 1978). SpoIIID was excised from the gel, electroeluted, acetone precipitated, and dissolved in phosphate-buffered saline (Harlow & Lane, 1988). 10 μg was emulsified with Freund's complete adjuvant (BRL) and injected into or near the popliteal gland of a New Zealand White rabbit. After a period of 3 weeks, the rabbit received at the same location a booster injection containing 5 μg of SpoIIID emulsified in Freund's incomplete adjuvant (BRL). One week later, the rabbit was bled and serum was prepared (Harlow & Lane, 1988).

(d) *Western blot analysis*

Every hour after the onset of sporulation, cells were harvested by centrifugation (14,000 *g* for 5 min) and whole-cell extracts were prepared as described previously (Healy *et al.*, 1991) except the lysis buffer did not contain DNase I. The amount of protein present in the extracts was quantified by the Bradford method (Bradford, 1976). After the addition of 0·5 vol. 3× sample buffer, proteins were separated by SDS/PAGE (18% polyacrylamide gel; Thomas & Kornberg, 1978) and electroblotted to a poly(vinylidene difluoride) membrane (Matsudaira, 1987). The membrane was incubated in blocking buffer (20 mM-Tris·HCl, pH 7·5, 0·5 M-NaCl, 2% nonfat dry milk) for 2 h at room temperature with shaking in order to block non-specific interactions between the primary antibodies and the membrane. The membrane was then probed for at least 2 h with shaking at room temperature with either polyclonal antiserum prepared against SpoIIID (this study) or polyclonal antiserum prepared against pro-σ^K (characterized previously; Lu *et al.*, 1990) diluted 1:1000 in antibody buffer (20 mM-Tris·HCl, pH 7·5, 0·5 M-NaCl, 2% nonfat dry milk, 0·05% Tween 20). Immunodetection utilizing goat anti-rabbit alkaline phosphatase conjugate was performed following the manufacturer's instructions (Bio-Rad). Signals were quantified using a Visage Digital Imager. All signals that were quantified were in the linear response range of the imager as determined by scanning the signals produced by different amounts of gel-purified SpoIIID in a Western blot experiment utilizing the anti-SpoIIID antibodies (data not shown).

(e) *β-Galactosidase assays*

β-Galactosidase activity was determined using the substrate *o*-nitrophenol-*β*-D-galactoside (ONPG) as described previously (Miller, 1972). One unit of enzyme hydrolyzes 1 μmol of substrate/min per A_{595} of initial cell density.

(f) *Northern blot analysis*

At hourly intervals between 3 and 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 (Igo & Losick, 1986) 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, then the RNA was fractionated by electrophoresis on a 1·2% (w/v) agarose gel containing 1·11% (v/v) formaldehyde, transferred to nitrocellulose, and hybridized at 55°C to nick-translated pBK39 (Kunkel *et al.*, 1989) as described (Ausubel *et al.*, 1989). The signals were visualized by autoradiography.

3. Results

(a) *The level of SpoIIID changes during sporulation*

Figure 1A shows the level of SpoIIID in wild-type cells harvested at hourly intervals during sporulation. We measured the level of SpoIIID in whole-cell extracts by using antibodies generated against SpoIIID in Western blot analysis. The anti-SpoIIID antibodies detected three polypeptides in extracts from sporulating, wild-type cells (Fig. 1A). The SpoIIID polypeptide comigrated with gel-puri-

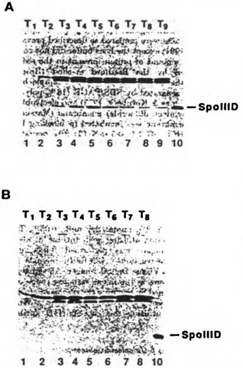

Figure 1. Characterization of anti-SpoIIID antibodies by Western blot analysis. Whole-cell extracts (5 μg) were prepared from cells collected at hourly intervals after the onset of sporulation in SM medium (lanes 1 to 9, respectively), and were subjected to Western blot analysis utilizing anti-SpoIIID antibodies. Gel-purified SpoIIID (10 ng) served as a positive control (lane 10). Panel A shows the wild-type Spo$^+$ strain PY79 and panel B shows the *spoIIID* mutant strain BK541 (*spoIIID*Δ*erm*), which has an erythromycin-resistance cassette replacing codons 15 to 37 of *spoIIID* (Kunkel *et al.*, 1989).

fied SpoIIID (Fig. 1A, lane 10) and was not detected in extracts prepared from sporulating, *spoIIID* mutant cells (Fig. 1B). SpoIIID was first detected three hours after the onset of sporulation (T_3), its level increased until T_5, and its level decreased sharply by T_6. This pattern is consistent with the pattern of β-galactosidase expression observed from a *spoIIID-lacZ* fusion during sporulation, except that the level of β-galactosidase remained constant after T_5 (Kunkel *et al.*, 1989).

The other two polypeptides detected by the anti-SpoIIID antibodies were larger than SpoIIID and were present in extracts prepared from both wild-type and *spoIIID* mutant cells. Apparently, the antibodies cross-react with two polypeptides that are not precursor or modified forms of SpoIIID, since these forms would be expected to be absent in the *spoIIID* mutant. These polypeptides are,

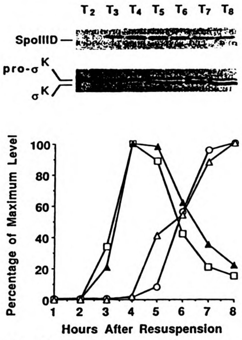

Figure 2. Levels of SpoIIID, pro-σ^K, σ^K and *cotD*-directed β-galactosidase activity in sporulating *B. subtilis*. The wild-type Spo$^+$ strain PY79 was lysogenized with phage SPβ::*cotD-lacZ* to construct strain BRH1. Whole-cell extracts (5 μg) were prepared from BRH1 cells collected at hourly intervals after the onset of sporulation in SM medium and were subjected to Western blot analyses utilizing anti-SpoIIID antibodies (upper inset) or anti-pro-σ^K antibodies (lower inset). The signals obtained were quantified using a Visage Digital Imager. β-Galactosidase activity reached a maximum level of 300 Miller units. The graph shows the level of SpoIIID (□), pro-σ^K (▲), σ^K (△), and *cotD*-directed β-galactosidase activity (○), each plotted as a percentage of the maximum level achieved during sporulation.

however, sporulation-specific, since these polypeptides were not detected in growing cells or in sporulating, *sigE* (encoding σ^E, a sporulation-specific sigma factor that functions earlier than σ^K; reviewed by Moran, 1989) mutant cells (data not shown).

(b) *The SpoIIID decrease coincides with increases in the level of σ^K and spore coat gene expression*

SpoIIID markedly represses transcription of the *cotD* promoter by σ^K RNA polymerase *in vitro* (Kroos *et al.*, 1989). The decrease in the level of SpoIIID after T_5 seemed to offer a possible explanation of how a repressive effect of SpoIIID on expression of *cotD in vivo* might be removed. Expression of *cotD* was shown previously to begin between T_3 and T_6 of sporulation, as determined by measuring the level of *cotD* mRNA or β-galactosidase activity from a *cotD-lacZ* fusion (Zheng & Losick, 1990). Also, the level of σ^K was shown previously to increase after T_4, although sporulation was induced differently than in the experiment shown in Figure 1 (Lu *et al.*, 1990). Figure 2 shows a direct comparison of the relationship between the SpoIIID decrease and the levels of *cotD* expression and σ^K. We collected samples of the wild-type strain carrying a *cotD-lacZ* fusion at hourly intervals during sporulation and measured the levels of SpoIIID, pro-σ^K, σ^K and *cotD*-directed β-galactosidase activity. The levels of pro-σ^K and σ^K were monitored by Western blot analysis using anti-pro-σ^K antibodies (Lu *et al.*, 1990). As the level of SpoIIID decreased fourfold between T_3 and T_7, *cotD*-directed β-galactosidase activity increased 12-fold. During the same interval the level of pro-σ^K decreased threefold and the level of σ^K increased twofold. Thus, there is a reciprocal relationship between the levels of σ^K and SpoIIID in sporulating cells; as the level of σ^K increases, the level of SpoIIID decreases. Both of these effects may play a role in the sharp rise in *cotD-lacZ* expression between T_3 and T_7. If SpoIIID does repress *cotD* transcription *in vivo*, as predicted from *in vitro* studies (Kroos *et al.*, 1989), these results suggest that a mechanism(s) exists to decrease the level of SpoIIID at the appropriate time during sporulation.

The results shown in Figure 2 are consistent with the idea that SpoIIID activates *sigK* transcription (Kunkel *et al.*, 1988; Kroos *et al.*, 1989). If SpoIIID activates *sigK* transcription, expression of *sigK* (as reflected by the total amount of pro-σ^K plus σ^K) would not be expected to increase after T_3, because the level of SpoIIID decreases sharply after T_3. As shown in Figure 2, the total amount of pro-σ^K and σ^K remained about the same after T_3. The level of pro-σ^K declined as the level of σ^K increased, presumably due to the processing of pro-σ^K to σ^K (Lu *et al.*, 1990).

(c) *Mutants defective in σ^K production are defective in the SpoIIID decrease*

The results shown in Figure 3 demonstrate that the production of active σ^K plays a role in the SpoIIID decrease. The level of SpoIIID was monitored in sporulation mutants shown previously to be defective in the production of σ^K (Lu *et al.*, 1990). Mutants that fail to produce pro-σ^K and σ^K, due either to a mutation in *sigK* (*spoIVCB* and *spoIIIC* encode the N-terminal and C-terminal portions of σ^K, respectively; Stragier *et al.*, 1989) or a mutation in *spoIVCA* (encoding a recombinase that is essential for the chromosomal rearrangement which forms the composite *sigK* gene; Kunkel *et al.*, 1990; Sato *et al.*, 1990) accumulated SpoIIID throughout sporulation (Fig. 3A). Mutants that produce pro-σ^K, but do not produce a detectable amount of σ^K, exhibited a partial (Fig. 3B, *spoIIIE*, *spoIIIG* and *spoIVB*) or delayed (Fig. 3C, *spoIIIA* and *spoIVF*) decrease in the level of SpoIIID during sporulation. These mutants may produce a low level of σ^K that cannot be detected by Western blot analysis using anti-pro-σ^K antibodies (Lu *et al.*, 1990), but is sufficient to elicit a partial or delayed decrease in the

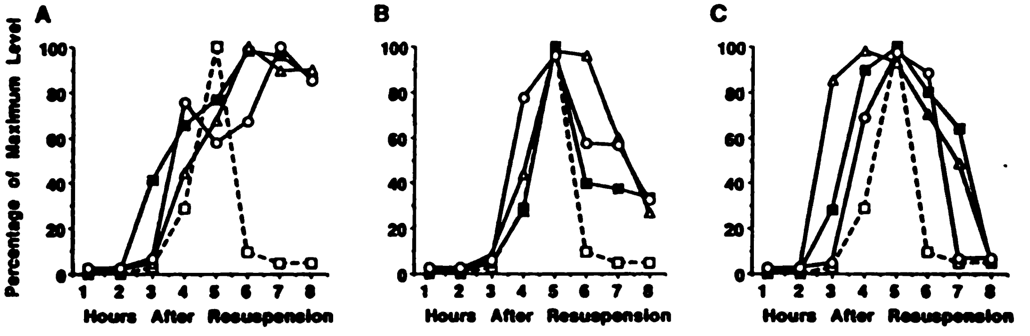

Figure 3. Level of SpoIIID in sporulation mutants with defects in σ^K production. Whole-cell extracts (5 μg) were prepared from cells collected at hourly intervals after the onset of sporulation in SM medium and were subjected to Western blot analyses utilizing anti-SpoIIID antibodies. For each mutant, the experiment was repeated at least once and in all cases changes in the level of SpoIIID during sporulation were qualitatively reproducible. A representative blot for each mutant was chosen and the SpoIIID signal was quantified using a Visage Digital Imager. Visible signals below the sensitivity of the imager were assigned a value of 5%. For each mutant, the SpoIIID level at each time point is plotted as a percentage of the maximum level achieved during sporulation. The maximum level of SpoIIID achieved was similar in all mutants except for *spoIIIC94*, in which SpoIIID reached a 3-fold higher level. For comparison, the level of SpoIIID in the wild-type Spo$^+$ strain PY79 (□) is shown in each panel. A, SpoIIID remained at a high level in strains BK410 (*spoIIIC94*; ■), BK558 (*spoIVCA133*; △) and KS815 (*spoIVCB*::Tn*917Hu215*; ○) B, SpoIIID decreased partially in strains SC768 (*spoIIIGΔ1 cat*; △), SC622 (*spoIIIE36*; ■) and BK750 (*spoIVBΔerm*; ○). C, The SpoIIID decrease was delayed in strains KS13 (*spoIIIA*::Tn*917Hu13*; ■), SC834 (*spoIVFB152*; △) and KS194 (*spoIVA*::Tn*917Hu194*; ○). These mutant strains have been described previously (Sandman *et al.*, 1987; Kunkel *et al.*, 1989; Cutting *et al.*, 1990, 1991*a*; Lu *et al.*, 1990).

level of SpoIIID. Consistent with this idea is the observation that a *spoIVA* mutant, which produces a low level of σ^K that is barely detectable by Western blot analysis (Lu *et al.*, 1990), exhibited a slightly delayed decrease in the level of SpoIIID (Fig. 3C) Also, the *spoIIIA*, *spoIIIG*, *spoIVB* and *spoIVF* mutants do retain the capacity to produce σ^K, because when pro-σ^K is overexpressed from a multicopy plasmid in these mutants, a low level of σ^K (detectable by Western blot analysis) is produced and the σ^K-transcribed *cotD* gene is expressed (S. Lu & L. Kroos, unpublished results). Alternatively or in addition, the pro-σ^K produced in these mutants may affect the SpoIIID level. Clearly, all mutants tested that were shown previously to be defective in σ^K production were shown here to be defective in the SpoIIID decrease. Taken together, these results strongly support the hypothesis that in wild-type cells the production of active σ^K causes the SpoIIID decrease.

(d) *The SpoIIID decrease occurs earlier in cells that produce σ^K earlier*

If the SpoIIID decrease is caused by the appearance of σ^K, then earlier production of σ^K should result in an earlier decrease in the level of SpoIIID. Figure 4A shows that the level of SpoIIID began to decrease about one hour earlier than normal in a mutant engineered to express σ^K earlier than normal (compare Fig. 4A to Fig. 2). We monitored the level of SpoIIID in a mutant (*spoIVCB*Δ*19*; Cutting *et al.*, 1990) expected to produce σ^K earlier during sporulation than the wild-type strain. The *spoIVCB*Δ*19* mutation deletes codons 2 to 20 of *spoIVCB*, which encodes the N terminus of pro-σ^K (Stragier *et al.*, 1989). After the chromosomal rearrangement generates the composite *sigK* gene in this strain, the primary translation product is active σ^K rather than pro-σ^K. Samples from the *spoIVCB*Δ*19* mutant carrying a *cotD-lacZ* fusion were collected every hour after the onset of sporulation and assayed for SpoIIID, σ^K and *cotD*-directed β-galactosidase activity. σ^K was produced about one hour earlier than normal in the *spoIVCB*Δ*19* mutant; σ^K was first detected at T_3 and reached a maximum level at T_4 (Fig. 4A), as was observed for pro-σ^K in the wild-type strain (Fig. 2). The SpoIIID level decreased about one hour earlier in the *spoIVCB*Δ*19* mutant than in wild-type cells. As documented in Figure 4A, and as was observed in three additional experiments (data not shown), the level of SpoIIID reproducibly decreased about twofold between T_4 and T_5 in the *spoIVCB*Δ*19* mutant. In wild-type cells, the level of SpoIIID reproducibly decreased from a high level at T_5 to less than half the maximal level at T_6, as documented in Figure 2, and as was observed in three

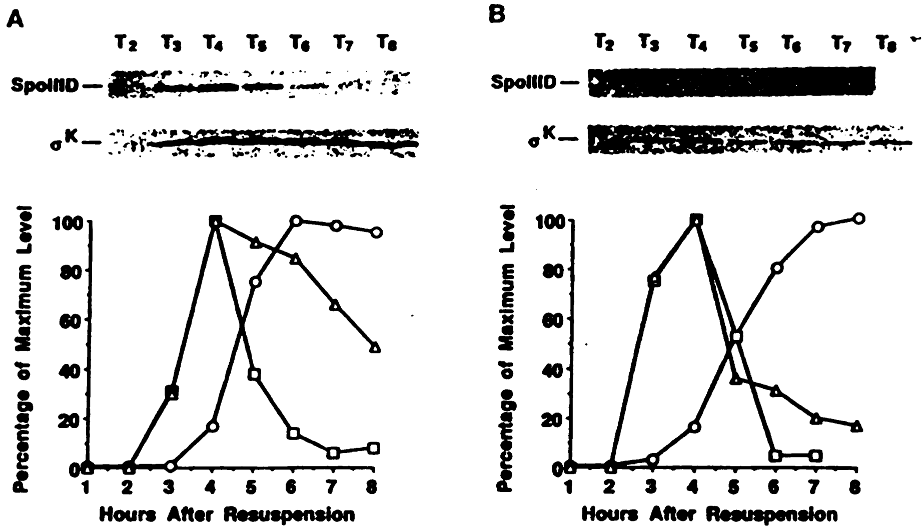

Figure 4. Levels of SpoIIID, σ^K and *cotD*-directed β-galactosidase activity in mutants that produce σ^K earlier than normal. The *B. subtilis* strains VO48 (*spoIVCB*Δ*19*; Cutting *et al.*, 1990) and BRH3 (*spoIVCB*Δ*19*, *gerE36*: see Materials and Methods) were lysogenized with phage SPβ::*cotD-lacZ* to construct strains BRH2 and BRH4, respectively. Whole-cell extracts (5 μg) were prepared from cells collected at hourly intervals after the onset of sporulation in SM medium and were subjected to Western blot analyses utilizing anti-SpoIIID antibodies (upper inset) or anti-pro-σ^K antibodies (lower inset). The signals obtained were quantified using a Visage Digital Imager. Visible signals below the sensitivity of the imager were assigned a value of 5%. β-Galactosidase activity reached maximum levels of 550 and 320 Miller units in strains BRH2 (A) and BRH4 (B), respectively. The graphs show the levels of SpoIIID (□), σ^K (△) and *cotD*-directed β-galactosidase activity (○), each plotted as a percentage of the maximum level achieved during sporulation.

additional experiments (data not shown). In addition, the earlier production of σ^K in the *spoIVCBΔ19* mutant appears to limit SpoIIID production, since the maximum level of SpoIIID was lower in the mutant (Fig. 4A) than in wild-type cells (Fig. 2). These results support the idea that the production of active σ^K causes the SpoIIID decrease.

Figure 4A also shows that the time of *cotD-lacZ* induction was about one hour earlier in the *spoIVCBΔ19* mutant than in wild-type cells (compare Fig. 4A to Fig. 2). In both cases, an increase in *cotD-lacZ* expression coincided with a decrease in the level of SpoIIID. However, *cotD*-directed β-galactosidase activity lagged considerably behind σ^K production in the *spoIVCBΔ19* mutant (Fig. 4A), whereas only a slight lag was observed in wild-type cells (Fig. 2). SpoIIID-mediated repression of *cotD* transcription might explain these observations, but we also considered the possibility that the lag represents time required for σ^K-directed synthesis of GerE, a DNA-binding protein that in wild-type cells greatly stimulates *cotD* expression (Cutting & Mandelstam, 1986; Cutting *et al.*, 1989; Zheng & Losick, 1990; Zheng *et al.*, 1992).

Figure 4B shows that GerE synthesis does not account for the lag between σ^K production and *cotD-lacZ* expression in the *spoIVCBΔ19* mutant. We constructed a *spoIVCBΔ19, gerE36* double mutant and monitored the levels of SpoIIID, σ^K and *cotD*-directed β-galactosidase activity during sporulation. The *gerE* mutation did not affect the time of *cotD-lacZ* induction, which still lagged considerably behind σ^K production (compare Fig. 4B to Fig. 4A). However, the major increase in *cotD-lacZ* expression did coincide with the SpoIIID decrease in the *spoIVCBΔ19, gerE36* double mutant (Fig. 4B), which is consistent with the idea that the SpoIIID decrease derepresses *cotD* transcription *in vivo*.

(e) *Production of* σ^K *during sporulation leads to a decrease in the level of* spoIIID *mRNA*

The decrease in the level of SpoIIID during the later stages of sporulation could, at least in part, be due to a decrease in the level of *spoIIID* mRNA available for translation. Figure 5 shows that the level of *spoIIID* mRNA decreased sharply between five and six hours after the onset of sporulation in wild-type cells, but not in *sigK* mutant cells. We measured the level of *spoIIID* mRNA in sporulating wild-type and *sigK* mutant cells using Northern blot analysis. The probe in the experiment was pBK39, which contains a 1·1 kb† fragment of *B. subtilis* DNA centered about the *spoIIID* coding sequence (Kunkel *et al.*, 1989). Similar results were obtained when a 306 bp *Apa*LI-*Xmn*I DNA fragment extending from the first codon of *spoIIID* to 25 bp beyond the translational stop codon of *spoIIID* served as the probe. An RNA of approximately 1340 bases was detected in RNA isolated from sporulating, wild-type cells (lanes 1 to 5) and this RNA was not detected in RNA isolate from a *spoIIID* mutant bearing an insertion of a drug resistance gene near the 5′ end of *spoIIID* (lanes 11 and 12). Therefore, we believe the 1340 base RNA is the mRNA transcript derived from *spoIIID*. Based on the transcript size and the position of the transcriptional start site (Tatti *et al.*, 1991), the transcript is predicted to extend about 830 bases beyond the end of the *spoIIID* coding sequence, suggesting the possibility of an additional downstream gene(s) at the *spoIIID* locus.

Figure 5. Level of *spoIIID* mRNA in sporulating wild-type and *sigK* mutant cells. RNA (20 μg) was prepared from cells collected at the indicated times after the onset of sporulation in SM medium and was analyzed by Northern blot analysis. *B. subtilis* strain PY79 was the wild-type Spo⁺ strain (lanes 1 to 5), BK410 (*spoIIIC94*; Kunkel *et al.*, 1989) was the *sigK* mutant (lanes 6 to 10) and BK541 (*spoIIIDΔerm*; Kunkel *et al.*, 1989) was the *spoIIID* mutant (lanes 11 and 12). The positions of RNA standards (0·16 to 1·77 kb RNA ladder; BRL) are indicated.

In sporulating, wild-type cells the level of *spoIIID* mRNA decreased sharply between T_5 and T_6 (Fig. 5, lanes 3 and 4). Thus, the level of *spoIIID* mRNA is markedly reduced late in sporulation and this could, at least in part, account for the decrease in the level of SpoIIID late in sporulation (Fig. 2). Figure 5 also shows that in sporulating *sigK* mutant cells, the level of *spoIIID* mRNA remained high throughout sporulation (lanes 6 to 10). The level of SpoIIID protein also remained high throughout sporulation in *sigK* mutant cells (Fig. 3). These results suggest that in wild-type cells σ^K negatively regulates the synthesis and/or stability of *spoIIID* mRNA and that this leads to a decrease in the level of SpoIIID late in sporulation.

4. Discussion

We have demonstrated that the level of SpoIIID in sporulating *B. subtilis* decreases at the appropriate time to produce a switch in the pattern of mother-cell gene expression from transcription of *sigK* at morphological stage IV to transcription of

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† Abbreviations used: kb, 10^3 base-pairs; bp, base-pair(s).

cotD at stage V. The following results strongly suggest that the decrease in the level of SpoIIID, and hence the switch, is controlled by the production of σ^K: (1) in wild-type cells the SpoIIID decrease coincides with an increase in the level of σ^K (Fig. 2); (2) mutants defective in σ^K production are defective in the SpoIIID decrease (Fig. 3); (3) in cells engineered to express σ^K earlier than normal, the SpoIIID decrease occurs earlier than normal and the maximum level of SpoIIID produced is lower than normal (Fig. 4); and (4) the level of *spoIIID* mRNA in *sigK* mutant cells remains high late in sporulation, whereas it decreases in wild-type cells (Fig. 5). Thus, production of active σ^K appears to initiate a feedback loop that leads to a decrease in the level of SpoIIID.

How does the negative feedback loop connecting σ^K production to the SpoIIID decrease operate? The production of σ^K normally leads to a decrease in the level of *spoIIID* mRNA late in sporulation (Fig. 5). Thus, σ^K appears to influence the rate of *spoIIID* transcription and/or the stability of the transcript. The observation that a *spoIIID-lacZ* fusion is overexpressed in mutants (*spoIIIA*, *spoIIIC*, *spoIIIG*, *spoIVCA* and *spoIVCB*; Kunkel *et al.*, 1989) that fail to make a detectable amount of σ^K (Lu *et al.*, 1990) suggests that the appearance of σ^K normally decreases the rate of *spoIIID* transcription.

Several lines of evidence indicate that *spoIIID* is transcribed by σ^E RNA polymerase (Kunkel *et al.*, 1989; Stevens & Errington, 1990; Tatti *et al.*, 1991). σ^E is a sporulation-specific sigma factor that functions earlier than σ^K (for a review, see Moran, 1989). σ^E appears to be a highly labile protein that is stabilized by binding to core RNA polymerase (Jonas *et al.*, 1990). Perhaps σ^K competes with σ^E for binding to core RNA polymerase, thereby destabilizing σ^E and decreasing transcription of *spoIIID*. Alternatively or in addition, σ^K RNA polymerase may direct transcription of a gene whose product represses *spoIIID* transcription directly or influences *spoIIID* transcription indirectly by affecting σ^E production. The decrease in the level of *spoIIID* mRNA between T_5 and T_6 (Fig. 5) is paralleled by a decrease in the level of SpoIIID protein (Fig. 2), suggesting that during this period of sporulation the mother cell is capable of rapidly degrading SpoIIID.

Figure 6 illustrates our current model for the switch from *sigK* to *cotD* transcription in the mother cell during the transition from morphological stage IV (cortex formation) of sporulation to stage V (coat formation). We propose that the σ^K RNA polymerase produced initially during stage IV is stimulated by SpoIIID to transcribe *sigK* and is prevented by SpoIIID from transcribing *cotD* (Fig. 6A). Also, during this period *gerE* would be transcribed by σ^K RNA polymerase (Cutting *et al.*, 1989; Zheng *et al.*, 1992). As the level of σ^K rises, it would cause a decrease in the level of SpoIIID and an increase in the level of GerE, eventually switching mother-cell gene expression to the stage V pattern (Fig. 6B). Because SpoIIID and GerE exert opposite effects on σ^K-directed transcription of *sigK* and *cotD* (Kroos *et al.*, 1989; Zheng *et al.*, 1992), a declining level of SpoIIID and a rising level of GerE would produce a reinforced switch from *sigK* to *cotD* transcription (Fig. 6).

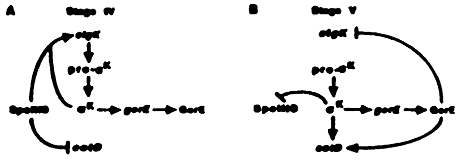

Figure 6. Model for the switch from *sigK* to *cotD* transcription in the mother cell during the stage IV to stage V transition of sporulation. A, During stage IV (cortex formation), SpoIIID stimulates *sigK* transcription by σ^K RNA polymerase and represses *cotD* transcription. σ^K RNA polymerase also transcribes *gerE*, leading to synthesis of GerE. B, The accumulation of σ^K, resulting from the processing of pro-σ^K to σ^K, causes a decrease in the level of SpoIIID, producing a switch to the stage V (coat formation) pattern of gene expression (i.e. *sigK* transcription is no longer stimulated and *cotD* transcription by σ^K RNA polymerase is no longer repressed). Continued production of GerE reinforces the switch since GerE represses *sigK* transcription and stimulates *cotD* transcription (Zheng *et al.*, 1992).

The model proposes that stage IV of sporulation is a period during which the σ^K RNA polymerase produced initially is stimulated by SpoIIID to transcribe *sigK* and is prevented by SpoIIID from transcribing *cotD*. This period could extend from between T_3 and T_4 to between T_4 and T_5 in wild-type cells, since σ^K is first detected at T_4 and *cotD*-directed β-galactosidase activity is first detected at T_5 (Fig. 2). SpoIIID reaches its maximum level during this period (Fig. 2), so it is available to affect transcription.

Evidence that SpoIIID stimulates *sigK* transcription by σ^K RNA polymerase comes from both *in vivo* and *in vitro* studies. Expression of a *sigK-lacZ* fusion is reduced in *sigK* mutant cells, demonstrating that σ^K does autoregulate its own expression, and *sigK-lacZ* expression is undetectable in *spoIIID* mutant cells, demonstrating that SpoIIID is essential for *sigK* transcription *in vivo* (Kunkel *et al.*, 1988). *In vitro*, SpoIIID greatly stimulates *sigK* transcription by σ^K RNA polymerase (Kroos *et al.*, 1989).

The suggestion that SpoIIID prevents *cotD* transcription by σ^K RNA polymerase for a short period during stage IV of sporulation is based primarily on the finding that SpoIIID can completely repress transcription of *cotD* by σ^K RNA polymerase *in vitro* (Kroos *et al.*, 1989). Consistent with this idea is the finding that an increasing level of *cotD*-directed β-galactosidase activity coincided with a decreasing level of SpoIIID in both wild-type cells (Fig. 2) and cells engineered to express σ^K earlier than normal (Fig. 4A). However, as illustrated in Figure 6B, *cotD* expression is stimulated by GerE (Zheng &

Losick, 1990; Zheng *et al.*, 1992). To eliminate the effect of GerE on *cotD-lacZ* expression, a *gerE* mutant was employed (Fig. 4B). In this strain, an increasing level of *cotD*-directed β-galactosidase activity still coincided with a decreasing level of SpoIIID, even though the level of σ^K was decreasing during the same period. This finding is consistent with the idea that SpoIIID represses *cotD* transcription *in vivo*. *In vitro* transcription studies also show that SpoIIID can repress transcription of *cotC* (encoding a spore coat protein; Donovan *et al.*, 1987) by σ^K RNA polymerase plus GerE (R. Halberg, H. Ichikawa & L. Kroos, unpublished results). Thus, SpoIIID may repress a set of spore coat genes for a short period during stage IV of sporulation.

SpoIIID does not inhibit transcription of the *gerE* promoter by σ^K RNA polymerase *in vitro* (L. Kroos & R. Losick, unpublished results). This result suggests that *gerE* expression would not be repressed by SpoIIID during stage IV (Fig. 6A). We find that *gerE* is expressed about one hour earlier during sporulation that *cotD*, as determined by measuring β-galactosidase activity from *lacZ* fusions or by Northern blot analysis of the mRNAs (our unpublished results). One gene encoding a spore coat protein, *cotA*, appears to be expressed with similar timing as *gerE*, as determined by measuring β-galactosidase activity from a *lacZ* fusion (our unpublished results). Although SpoIIID can inhibit *cotA* transcription *in vitro* by σ^K RNA polymerase (L. Kroos & R. Losick, unpublished results), this effect requires a higher molar ratio of SpoIIID to DNA than do the effects of SpoIIID on *sigK*, *cotD* and *cotC* transcription (our unpublished results). One or more genes involved in cortex formation may also be transcribed by σ^K RNA polymerase and not subject to repression by SpoIIID, since *sigK* mutants are defective in cortex formation (Cutting *et al.*, 1991*a*).

Our results suggest that as the level of σ^K rises during stage IV, it causes a decrease in the level of SpoIIID. As the amount of SpoIIID available to stimulate transcription of *sigK* (and perhaps other stage IV genes) by σ^K RNA polymerase and to repress transcription of *cotD* (and perhaps other stage V genes) diminishes, the pattern of mother-cell gene expression would switch to the stage V pattern (Fig. 6B). The results of Zheng *et al.* (1992) suggest that an increase in the level of GerE during stage IV would reinforce this switch in the pattern of mother-cell gene expression. because the effects of GerE on *sigK* and *cotD* transcription *in vitro* by σ^K RNA polymerase are just the opposite of the effects exerted by SpoIIID (Fig. 6). GerE can completely inhibit *sigK* transcription by σ^K RNA polymerase *in vitro* (Zheng *et al.*, 1992). *cotA* is also repressed by GerE (Sandman *et al.*, 1988; Cutting *et al.*, 1989; Zheng *et al.*, 1992). On the other hand, both *in vivo* and *in vitro* studies support the idea that GerE not only stimulates transcription of *cotD* by σ^K RNA polymerase (as shown in Fig. 6B), but it also stimulates σ^K-directed transcription of *cotB* and *cotC* during stage V (Zheng & Losick, 1990; Zheng *et al.*, 1992). Thus, both SpoIIID and GerE can positively or negatively affect σ^K-directed transcription of several different mother-cell-specific genes and the levels of SpoIIID, σ^K and GerE appear to be controlled by the regulatory interactions illustrated in Figure 6 so as to produce a molecular switch governing the stage IV to stage V transition of sporulation.

An interesting question is whether this switch is coupled to morphogenesis. *A priori*, it seems likely that mechanisms must exist to co-ordinate sporulation gene expression with morphogenic progress during development. Two such coupling points have been proposed previously. The proteolytic processing of pro-σ^E to σ^E may couple formation of the sporulation septum to the production of σ^E and the ensuing new pattern of gene expression (LaBell *et al.*, 1987; Stragier *et al.*, 1988). Similarly, several lines of evidence support the idea that proteolytic processing of pro-σ^K to σ^K is a regulatory device that couples forespore morphogenesis to the production of σ^K in the mother cell (Cutting *et al.*, 1990, 1991*b*; Lu *et al.*, 1990). Is the proposed switch governing the stage IV to stage V transition somehow coupled to the completion of cortex formation, or do the rates of synthesis and degradation of SpoIIID, σ^K and GerE constitute a developmental timer that determines the length of stage IV? These possibilities are not mutually exclusive. For example, the rate(s) of synthesis and/or degradation of SpoIIID, σ^K and/or GerE may be responsive to a signal generated upon completion of the spore cortex. This would be analogous to the mechanism controlling the decision of bacteriophage λ to lyse or lysogenize its *E. coli* host, where regulatory interactions between several regulatory proteins produce a molecular switch (Ptashne, 1987) and degradation of the cII protein is affected by viral, host and environmental factors (Herskowitz & Hagen, 1980; Hoyt *et al.*, 1982).

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Note added in proof. The level of *spoIIID* mRNA remains high late in sporulation (i.e. between T_3 and T_6) in *spoIVCA* mutant cells (strain BK558 (*spoIVCA133*); Kunkel *et al.*, 1989), which fail to produce σ^K, supporting the idea that σ^K production negatively regulates the synthesis and/or stability of *spoIIID* mRNA.

CHAPTER IV

The SpoIIID Switch Protein Is Converted to a 9 kDa Form During *Bacillus subtilis* Sporulation

"There is nothing permanent except change."

Heraclitus

Abstract

Mother-cell-specific gene expression during sporulation of *Bacillus subtilis* is, in part, controlled by σ^K RNA polymerase. σ^K is required for the formation of the spore cortex, stage IV, and the formation of the spore coat, stage V. It has been demonstrated that SpoIIID stimulates the transcription of a stage IV gene, but represses the transcription of several stage V genes by σ^K RNA polymerase *in vitro*. Based on these observations, it was proposed that the inactivation of SpoIIID establishes a switch from the stage IV to stage V pattern of gene expression in the mother cell. Consistent with this idea, the level of SpoIIID decreases sharply at the appropriate time during sporulation. Here we provide evidence that this decrease involves the conversion of SpoIIID to a less stable 9 kDa form. We demonstrate that this conversion is developmentally regulated. The conversion of SpoIIID to the 9 kDa protein may be important to relieve the repressive effect that SpoIIID has on the expression of several stage V genes.

Introduction

Under conditions of nutrient deprivation the gram-positive bacterium *Bacillus subtilis* undergoes a series of morphological changes that culminate in the formation of a mature spore (reviewed by (Errington, 1993)). The first easily observed morphological change is an asymmetrically positioned septum, which divides the bacterium into two compartments, the mother cell and the forespore. Each of these compartments receives a copy of the genome, but they realize alternative developmental fates because gene expression is regulated temporally and spatially. Spatial regulation is established by the compartment-specific activation of sigma subunits of RNA polymerase. Two mother-cell-specific sigma factors are σ^E (Driks & Losick, 1991) and σ^K (Kroos *et al.*, 1989; Stragier *et al.*, 1989). σ^E is required for the migration of the septum and engulfment of the forespore in a double membrane (stage III). σ^K is required for the deposition of cell-wall-like material called cortex between the membranes of the forespore (stage IV) (Cutting *et al.*, 1991a) and the synthesis of spore coat proteins that assemble on the surface of the forespore (stage V) (Kroos *et al.*, 1989; Zheng & Losick, 1990).

σ^K is initially synthesized as an inactive precursor, pro-σ^K, which has an additional 20 amino acids at its N-terminus (Kroos *et al.*, 1989; Stragier *et al.*, 1989; Lu *et al.*, 1990). Proteolytic processing of pro-σ^K to σ^K is dependent upon forespore-specific gene expression (Cutting *et al.*, 1990; Lu *et al.*, 1990). Thus, the processing event appears to couple gene expression in both compartments.

σ^K-dependent transcription is affected by SpoIIID, a 10.8 kDa DNA-binding protein [Kunkel *et al.*, 1989; Stevens & Errington, 1990; Chapter II]. For example, SpoIIID stimulates *sigK* (encoding pro-σ^K) transcription, but represses *cotC*, *cotD*, and *cotX* (encoding spore coat proteins) transcription by σ^K RNA polymerase *in vitro* (H. Ichikawa, R. Halberg, L. Kroos, unpublished data; H. Ichikawa and L. Kroos, unpublished data; Kroos *et al.*, 1989). Previously, it was proposed that the inactivation and/or sequestering of SpoIIID establishes a switch in the mother-cell pattern of gene expression from the transcription of *sigK* at stage IV to the transcription of spore coat genes at stage V (Kroos & Losick, 1989). Consistent with this idea, the level of SpoIIID decreases at the appropriate time during sporulation (Halberg & Kroos, 1992). This decrease is dependent upon the processing of pro-σ^K to σ^K (Halberg & Kroos, 1992). The accumulation of σ^K reduces the level of *spoIIID* mRNA with similar timing during sporulation as the reduction in the level of the SpoIIID protein (Halberg & Kroos, 1992).

The parallel decrease in the levels of *spoIIID* mRNA and SpoIIID suggests that a mechanism exists for degrading SpoIIID in sporulating cells. Here we provide evidence that SpoIIID is converted to a less stable 9 kDa form by removing 7 amino acids from its C-terminus. We demonstrate that this conversion is developmentally regulated. The conversion of SpoIIID to the 9 kDa protein may be necessary to relieve the repressive effect that SpoIIID has on the expression of spore coat genes during sporulation.

Materials and Methods

Bacterial Strains

B. subtilis strains were provided by R. Losick at Harvard University. All strains were isogenic with PY79 (Youngman *et al.*, 1984) except for 91 (*spoVB91;* Piggot and Coote, 1976), SC50 (*spoVC134;* Coote, 1972), and SC53 (*spoVF224;* Coote, 1972).

Growth and Sporulation

Sporulation was induced by resuspending growing cells in SM medium as described previously (Sterlini & Mandelstam, 1969). The onset of sporulation (T_0) is defined as the time of resuspension.

Matrix-Assisted Laser Desorption Ionization (MALDI) Mass Spectrometric Analysis

SpoIIID and the 9 kDa protein were partially purified from sporulating *B. subtilis* following the procedure for partially purifying SpoIIID and σ^K RNA polymerase described previously (Kroos *et al.*, 1989). Proteins in DNA-cellulose column fractions were precipitated in 10% trichloroacetic acid, resuspended and boiled 5 minutes in sample buffer (0.125 M Tris·HCl, pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue), and subjected to SDS-PAGE (18% polyacrylamide; (Thomas

& Kornberg, 1978)). SpoIIID and the 9 kDa protein (approximately 10 μg each) were excised from the gel, eluted following the procedure described previously (Hager & Burgess, 1980) except the elution buffer did not contain BSA, acetone precipitated, and dissolved in 1 μl of 6 M guanidine·HCl and 19 μl of 0.1% trifluoroacetic acid (TFA). The samples (2 μl) were applied to membranes (Zetabind; 0.45 μm pore size and 50 μm thickness) attached to a stainless steel probe tip. The sample was allowed to air dry and then the membrane was washed by immersion in deionized water for 15-20 seconds at room temperature.

The SpoIIID digest with endoproteinase Asp-N was performed as follows. SpoIIID was applied to membrane attached to a probe tip as described above. The probe tip was placed in a glass vial containing a small amount of 100 mM ammonium bicarbonate, pH 8.0. Endoproteinase Asp-N (2 μl of 0.04 μg/μl) was applied to the membrane and the glass vial was sealed. The digest was incubated 14 hours at 25°C.

To analyze the samples, a saturated solution of α-cyano-4-hydroxycinnamic acid in 1:1 acetonitrile/0.1% TFA (2 μl) was applied to the membrane and air dried. Mass spectra were obtained utilizing a VT-200 linear time of flight mass spectrometer equipped with a nitrogen laser (337 nm, 3 ns pulse) as described previously (Zaluzec *et al.*, 1994).

DNase I Footprinting

DNA probes labelled at only one end were prepared as follows. For the analysis of *sigK*, 20 μg of pBK16 (Kroos *et al.*, 1989) was digested with *Xba*I and labelled by treating with alkaline phosphatase followed by phage T4 polynucleotide kinase and (γ-^{32}P) ATP. The labelled DNA was digested with *Pst*I and the 352 bp *Xba*I-*Pst*I fragment was purified after electrophoresis in a non-denaturing polyacrylamide gel using the crush and soak method described previously (Sambrook *et al.*, 1989), except 20 μg of double-stranded poly (dI·dC) (Pharmacia) was added to serve both as a carrier during ethanol precipitation and as a competitor during footprinting experiments. Similar end-labelling and recovery methods were used to prepare a DNA fragment for the analysis of *cotD*. A 324 bp *Eco*RI-*Taq*I fragment from pLRK100 (Kroos *et al.*, 1989) was labelled at the *Eco*RI site.

DNase I footprinting experiments were performed according to method 2 described (Zheng *et al.*, 1992), except 0.5 pmole of end-labelled DNA was used. SpoIIID and the 9 kDa protein were gel-purified following the procedure described for SpoIIID previously (Kroos *et al.*, 1989).

In vitro Transcription

σ^E RNA polymerase was a gift from Kathleen Tatti and Charlie Moran at Emory University. σ^K RNA polymerase was partially purified from *gerE* mutant cells (SC104; *gerE36*; Kroos *et al.*, 1989), following the procedure described previously (Kroos *et al.*, 1989). The σ^K RNA polymerase was comparable in protein composition and in *cotD*- and *sigK*-transcribing activities to fraction 24 shown in Figure 2 of (Kroos *et al.*, 1989). Transcription reactions (45 µl) were performed as described previously (Carter & Moran, 1986), except that RNA polymerase was allowed to bind to the DNA template for 10 minutes at 37°C before the addition of nucleotides. The labelled nucleotide was (α-^{32}P) CTP. Heparin (6 µg) was added 2 minutes after the addition of nucleotides to prevent reinitiation. After the reactions were stopped, 20 µl of each reaction mixture was subjected to electrophoresis and transcripts were detected by autoradiography.

Mobility Shift Assays

The DNA probe was either a synthetic oligonucleotide or a 106 bp *Eco*47III-*Xba*I DNA fragment prepared from pBK16 (Kroos *et al.*, 1989), containing the SpoIIID binding sites in the *sigK* open reading frame (Chapter II). The former was prepared as follows. Two complementary oligonucleotides

(AGATACTAAAAAGACAAGCTCTTT and GTTAAAGAGCTTGTCTTTTTAGTA) were synthesized (MSU Macromolecular Structure Facility) and combined in annealing buffer (67 mM Tris·HCl, pH 7.6, 13 mM $MgCl_2$, 6.7 mM DTT, 1.3 mM spermidine, and 1.3 mM EDTA). The mixture was incubated at 88°C for 2 minutes, 65°C for 10 minutes, 37°C for 10 minutes, and room temperature for 5 minutes. The double-stranded oligonucleotide was labelled by treating with T4 polynucleotide kinase and (γ-^{32}P) ATP. The 106 bp *Eco*47III-*Xba*I fragment was prepared as follows. pBK16 (Kroos *et al.*, 1989) was digested with *Hind*III and *Xba*I. The DNA fragments were labelled by treating with alkaline phosphatase followed by T4 polynucleotide kinase and (γ-^{32}P) ATP. The labelled DNA was digested with *Eco*47III.

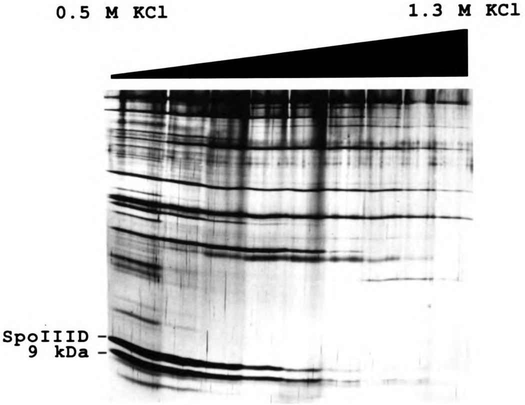
B. subtilis cells (1 ml) were harvested at hourly intervals after the onset of sporulation by centrifugation (14,000*g* for 5 minutes at room temperature). The supernatant was removed. Cell pellets were quickly frozen in a dry ice/ethanol bath and stored at -80°C. Extracts were prepared by resuspending cell pellets in 200 µl of Buffer I (Shorenstein & Losick, 1973) supplemented with 5% (v/v) phenylmethylsulfonyl fluoride (PMSF) (6 mg/ml in 95% ethanol), sonicating the cells, and centrifuging the lysate (14,000*g* for 2 minutes at 4°C). The protein concentration of the extracts was determined by Bradford analysis (Bradford, 1976).

The DNA probes and a 10-fold excess of double-stranded poly (dI·dC) (Pharmacia) were combined with extracts, gel-purified SpoIIID, or gel-purified 9 kDa protein in binding buffer (10 mM Tris·HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA and 5% (v/v) glycerol). The mixtures were incubated for 20 minutes at 37°C and electrophoresed on non-denaturing polyacrylamide gels. The unbound DNA and DNA·protein complexes were visualized by autoradiography.

Results

A 9 kDa protein that copurifies with SpoIIID appears to be a degradation product of SpoIIID. A decrease in the level of *spoIIID* mRNA is paralleled by a decrease in the level of SpoIIID, suggesting a mechanism for degrading SpoIIID exist in sporulating *B. subtilis* (Halberg & Kroos, 1992). A clue to how this mechanism may work was obtained during the purification of SpoIIID. A 9 kDa protein co-eluted with SpoIIID upon salt-gradient elution of a double-stranded DNA-cellulose column (Figure 1). The first 20 amino acids of this protein were identical to those of SpoIIID, as determined by amino-terminal amino acid sequencing using sequential Edman degradation in an automated gas-phase sequentor (MSU Macromolecular Structure Facility; data not shown). This observation suggested that the 9 kDa protein may arise by removing amino acids from the C-terminus of SpoIIID. Consistent with this idea were the results of the mass spectral analysis of SpoIIID and the 9 kDa protein. Six independent experiments revealed that gel-purified SpoIIID consists of a single polypeptide with an average mass of 10813 ± 13 Da (Figure 2A; a representative spectrum), which is in good agreement with the predicted mass of SpoIIID (10803 Da; Kunkel *et al.* 1989). Digestion of gel-purified SpoIIID with endoproteinase Asp-N yielded seven peptides, which had masses in good agreement with the predicted masses

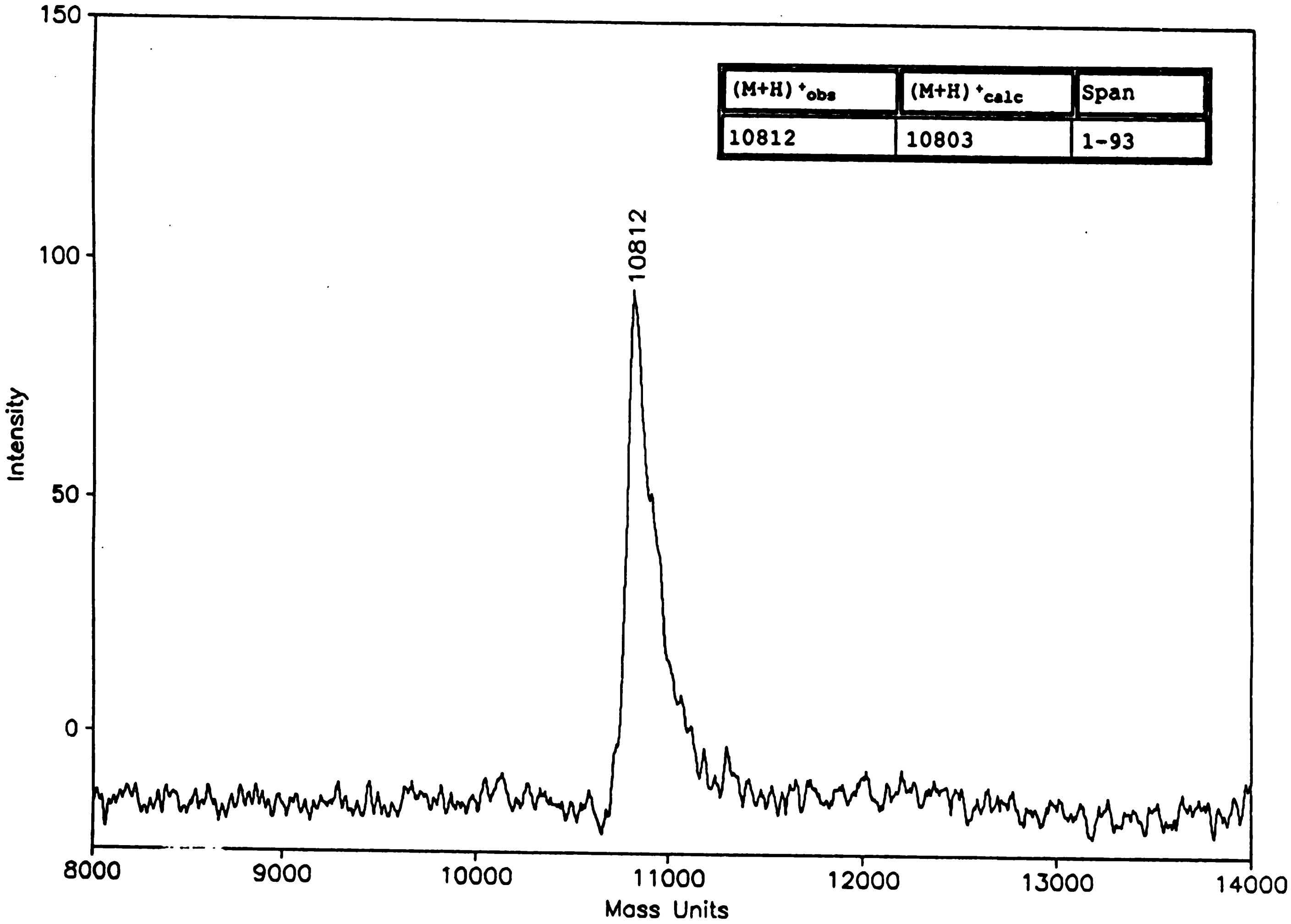
Figure 1. A silver-stained 18% polyacrylamide gel displaying proteins collected from a double-stranded DNA-cellulose column upon elution with a 0.5 M - 1.3 M potassium chloride gradient. The positions of SpoIIID and the 9 kDa protein are indicated.



0.5 M KCl
1.3 M KCl
SpoIIID
9 kDa

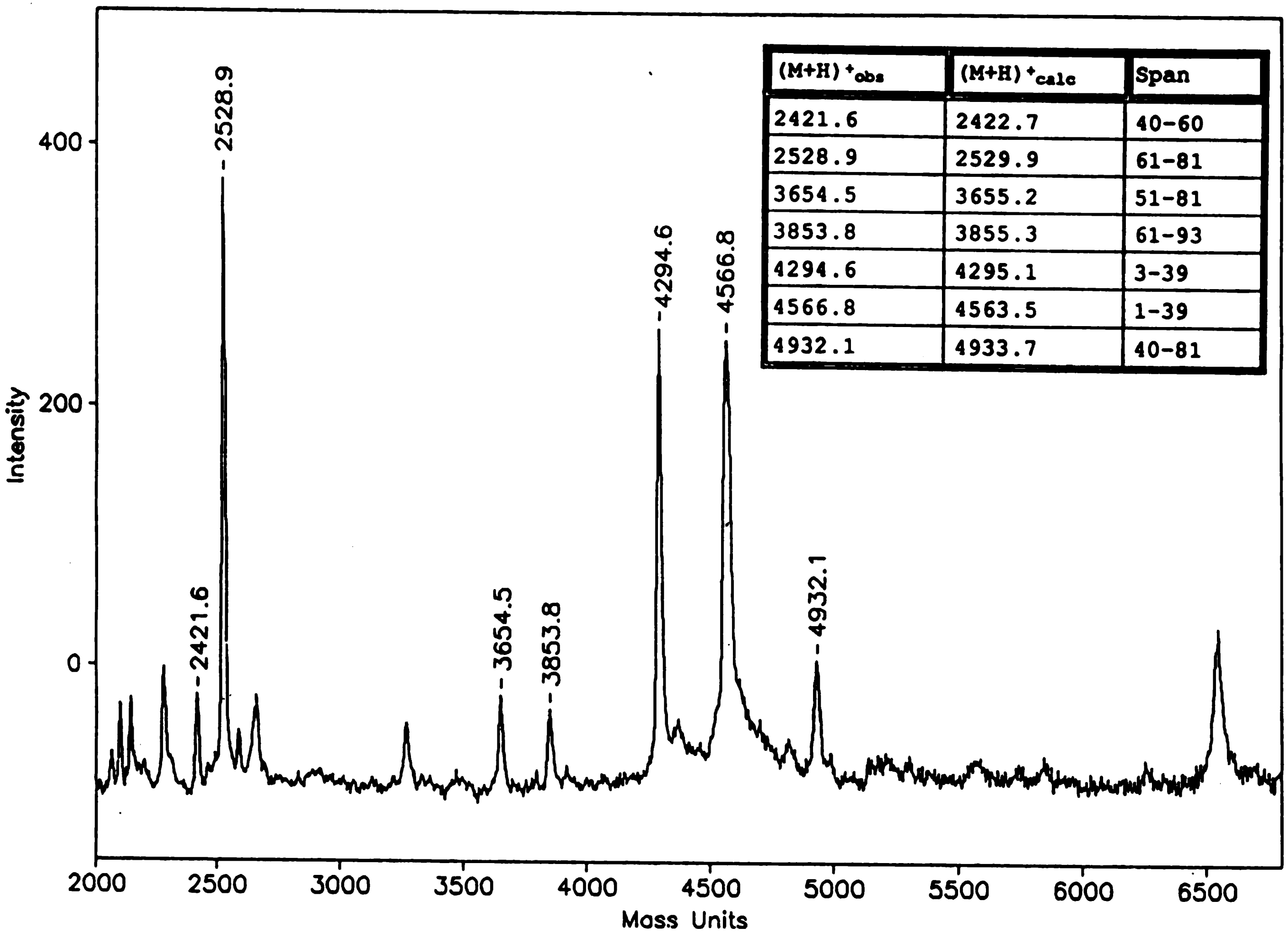
Figure 2. Mass spectra of SpoIIID and SpoIIID digested with endoproteinase Asp-N. Spectra of (A) SpoIIID and (B) SpoIIID digested with endoproteinase Asp-N were obtained by matrix-assisted laser desorption (MALDI) mass spectroscopy as described in the Materials and Methods. The intensity is in arbitrary units.

A



$(M+H)^+_{obs}$	$(M+H)^+_{calc}$	Span
10812	10803	1-93

B

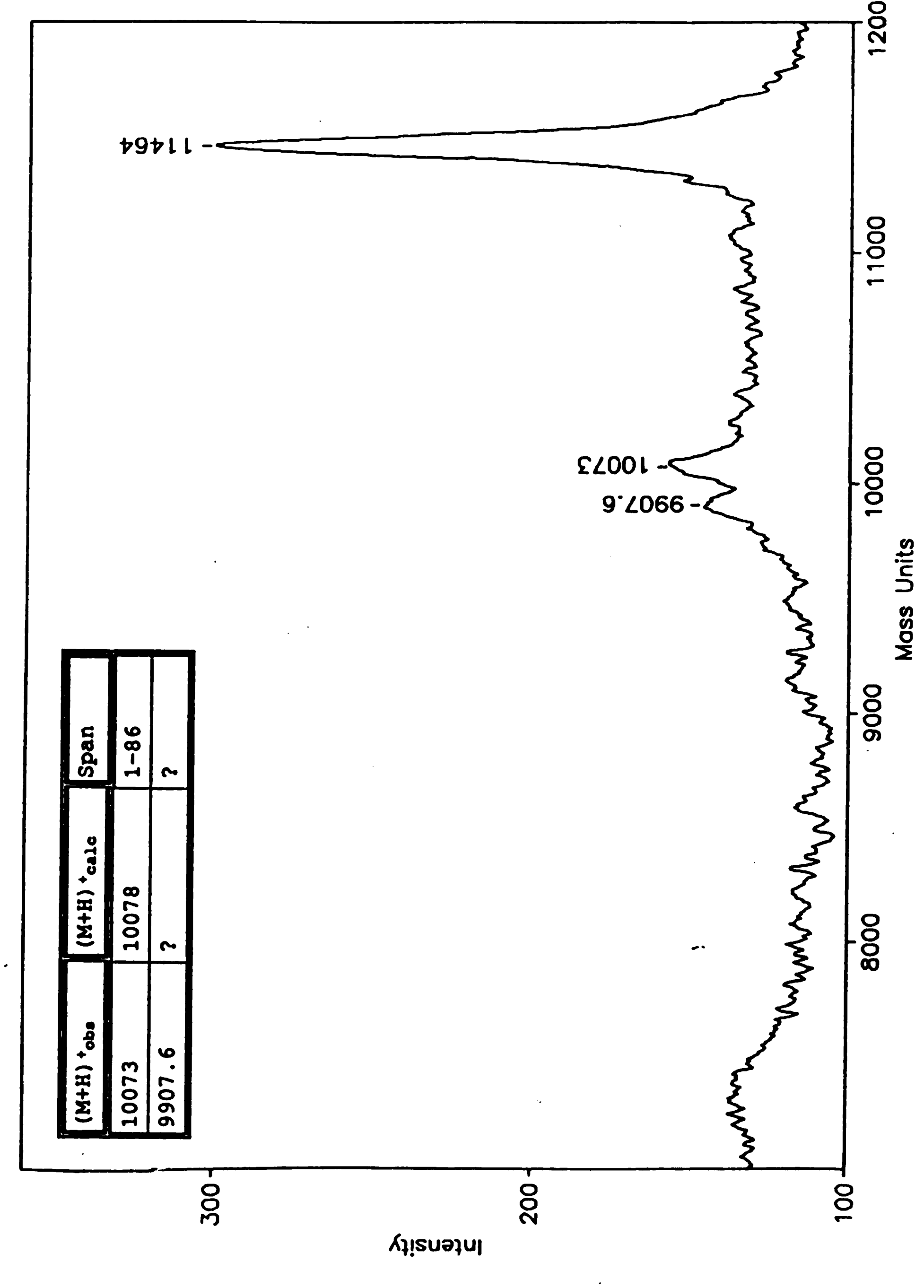


$(M+H)^+_{obs}$	$(M+H)^+_{calc}$	Span
2421.6	2422.7	40-60
2528.9	2529.9	61-81
3654.5	3655.2	51-81
3853.8	3855.3	61-93
4294.6	4295.1	3-39
4566.8	4563.5	1-39
4932.1	4933.7	40-81

of SpoIIID peptides expected to result from such a digest (Figure 2B). These fragments spanned the entire SpoIIID sequence (Figure 2B). Four independent experiments revealed that gel-purified 9 kDa protein consisted of two polypeptides with average masses of 10078 ± 5 Da and 9896 ± 15 Da (Figure 3; a representative spectrum). The relative amounts of these polypeptides varied from sample to sample. A mass of 10078 Da is in good agreement with the predicted mass (10073 Da) of a SpoIIID peptide spanning from amino acids 1 through 86 (i.e., missing the C-terminal 7 amino acids). A mass of 9896 Da does not match that of any predicted SpoIIID peptide, suggesting that it may be a contaminating protein. Taken together, N-terminal amino acid sequencing and mass spectral data suggest that SpoIIID is converted to a 9 kDa form by removing 7 amino acids from its C-terminus during sporulation. Additional experiments are required to determine the C-terminus of the 9 kDa protein with certainty.

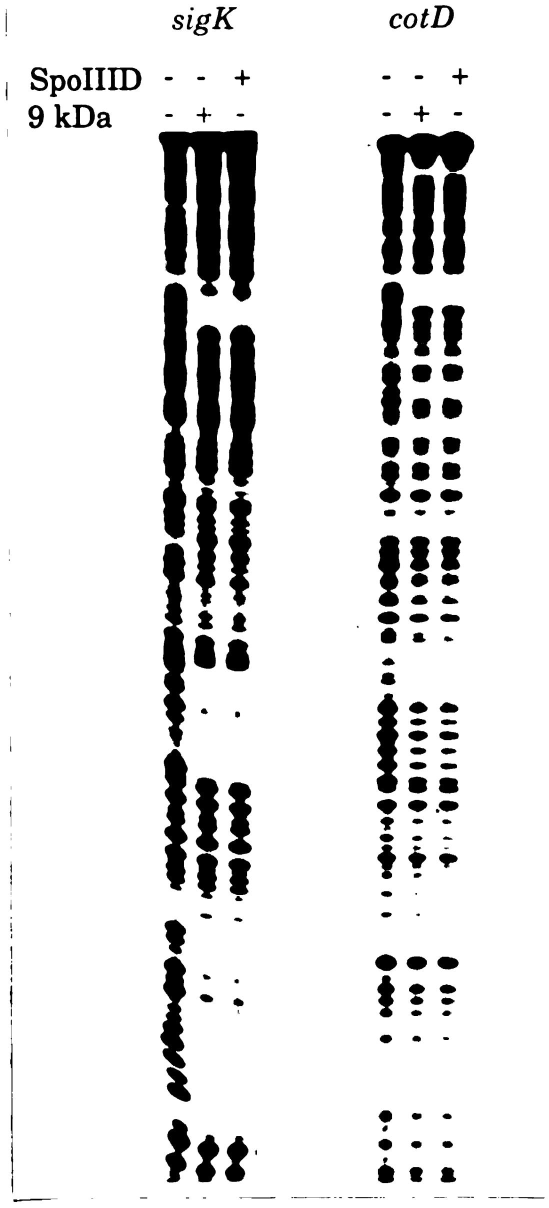
DNA-binding and transcriptional properties of the 9 kDa protein. The putative helix-turn-helix DNA-binding motif in SpoIIID spans from amino acid 23 to amino acid 42 (Kunkel *et al.*, 1989; Stevens & Errington, 1990). Based on this observation, the 9 kDa protein may, like SpoIIID (Chapter II), bind to *sigK* and *cotD*. To test this idea, DNase I footprinting experiments were performed (Figure 4). The 9 kDa protein protects the same regions of *sigK* and *cotD*

Figure 3. Mass spectrum of the 9 kDa protein. The spectrum was obtained by MALDI mass spectroscopy as described in the Materials and Methods. The observed mass at 11464 is $(2M+H)^+$ species of insulin (internal standard). The intensity is in arbitrary units.



$(M+H)^{+}_{obs}$	$(M+H)^{+}_{calc}$	Span
10073	10078	1-86
9907.6	?	?

Figure 4. SpoIIID and 9 kDa footprints on *sigK* and *cotD*. Radioactive DNA probes separately end-labelled were incubated in separate reactions with no protein, 120 ng gel-purified 9 kDa protein, or 120 ng gel-purified SpoIIID, and then digested with DNase I. The resulting DNA fragments were separated by electrophoresis on a 6% polyacrylamide gel containing 8 M urea. (A) Footprints in the *sigK* promoter region and open reading frame (ORF) identified using a probe labelled at the *Xba*I site located downstream of the transcriptional start site. (B) Footprints in the *cotD* promoter region and ORF identified using a probe labelled at the *Eco*RI site located upstream of the transcriptional start site.

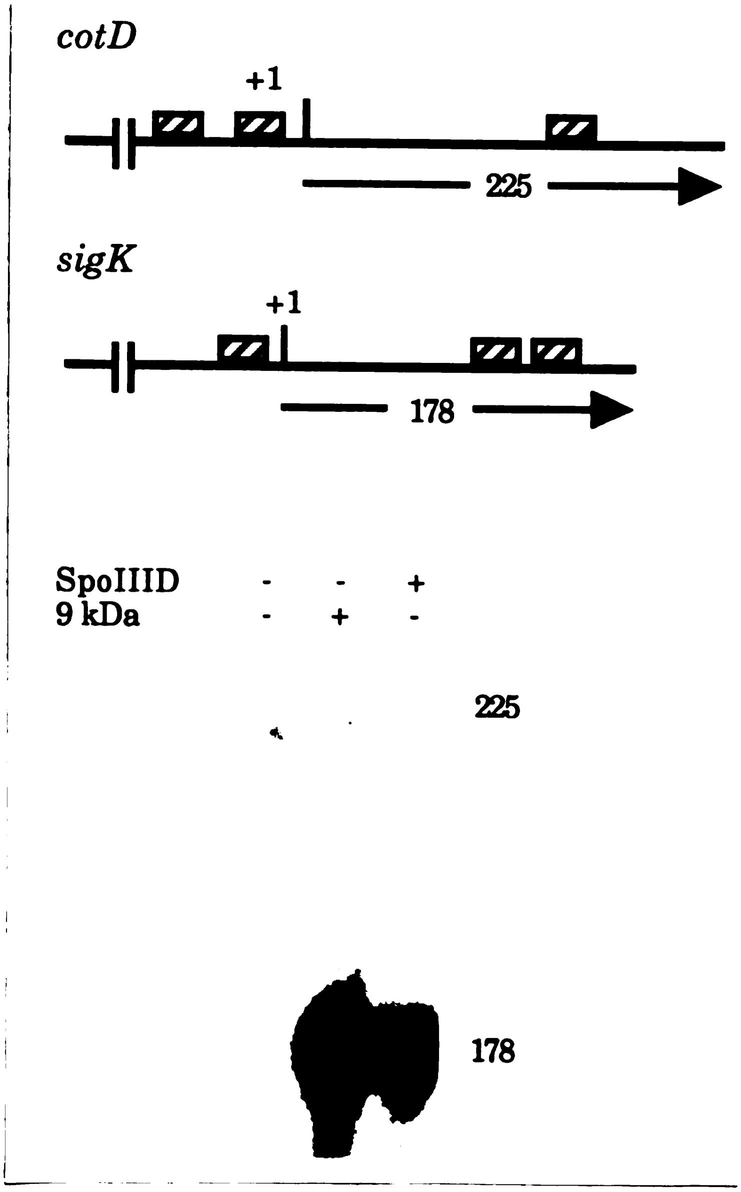

sigK
cotD
SpoIIID - - + - - +
9 kDa - + - - + -

from DNase I digestion as SpoIIID.

SpoIIID binding stimulates *sigK* transcription and represses *cotD* transcription by σ^K RNA polymerase *in vitro* (Kroos *et al.*, 1989). To test whether the 9 kDa protein affects the transcription of these genes, linearized DNA templates were transcribed by partially purified σ^K RNA polymerase alone or in the presence of gel-purified 9 kDa protein. The presence of the 9 kDa protein greatly activated *sigK* transcription, but failed to repress *cotD* transcription (Figure 5). This observation suggests that the C-terminus of SpoIIID is required for *cotD* repression. However, the gel-purified 9 kDa protein used in these experiments may have been contaminated with the 9896 Da polypeptide identified by mass spectral analysis. These experiments need to be repeated with more highly purified 9 kDa protein in order to determine unequivocally its DNA-binding and transcriptional properties.

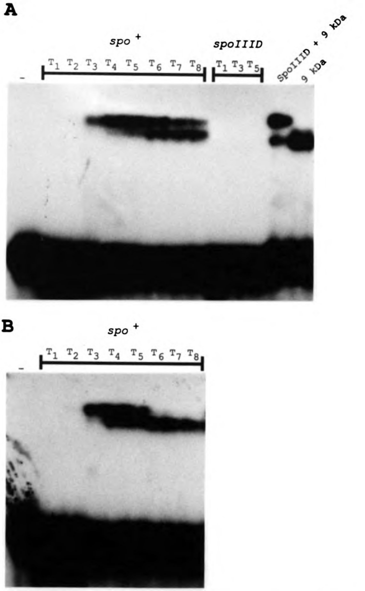
SpoIIID is converted to the 9 kDa protein in a developmentally regulated fashion. The 9 kDa protein was not detected in extracts of sporulating cells by Western blot analysis with polyclonal anti-SpoIIID antibodies (Halberg & Kroos, 1992); however, these antibodies recognized gel-purified 9 kDa protein weakly. Because the experiments presented above suggested that the 9 kDa protein would bind to specific sites in DNA with similar affinity as SpoIIID, we

Figure 5. Effects of SpoIIID and the 9 kDa protein on *sigK* and *cotD* transcription *in vitro*. DNA templates (1μg of pLRK100, which contains the *cotD* promoter, digested with *Hind*III and 1μg of pBK16, which contains the *sigK* promoter, digested with *Xba*I; Kroos et al., 1989) were transcribed with partially purified σ^K RNA polymerase (200 ng) alone (lane 1) or in the presence of either 120 ng of gel-purified 9 kDa protein (lane 2) or 120 ng of gel-purified SpoIIID (lane 3). Run-off transcripts were electrophoresed in a 5% polyacrylamide gel containing 8 M urea. Sizes of transcripts were estimated from the migration of end-labelled fragments of *Msp*I-digested pBR322.


cotD
+1
225
sigK
+1
178
SpoIIID - - +
9 kDa - + -
225
178

used a mobility shift assay to detect the 9 kDa protein in extracts of sporulating cells. Extracts prepared from cells harvested at hourly intervals between T_1 and T_8 were incubated with a radiolabelled DNA probe and then electrophoresed on a non-denaturing polyacrylamide gel. Two shifted complexes were observed with extracts prepared from wild-type cells (Figure 6A). The upper complex comigrated with a complex produced by gel-purified SpoIIID and DNA. This complex was first detected at T_3, its level increased until T_5, and its level decreased beginning at T_6. This pattern is consistent with the level of SpoIIID detected by Western blotting during sporulation (Halberg & Kroos, 1992). The lower complex comigrated with a complex produced by gel-purified 9 kDa protein and DNA. This complex was first detected at T_4 and its level increased until T_6. The upper and lower complexes were not observed in extracts prepared from *spoIIID* mutant cells (Figure 6A). Taken together, these results indicate that the upper and lower complexes correspond to binding of SpoIIID and the 9 kDa protein to the DNA probe, respectively. In addition, the absence of a 9 kDa protein complex in extracts of *spoIIID* mutant cells is consistent with the idea that the 9 kDa protein is derived from SpoIIID. If this is the case, the results shown in Figure 6A suggest that cells acquire the ability to convert SpoIIID to the 9 kDa protein beginning at T_4 of sporulation. The low abundance of the 9

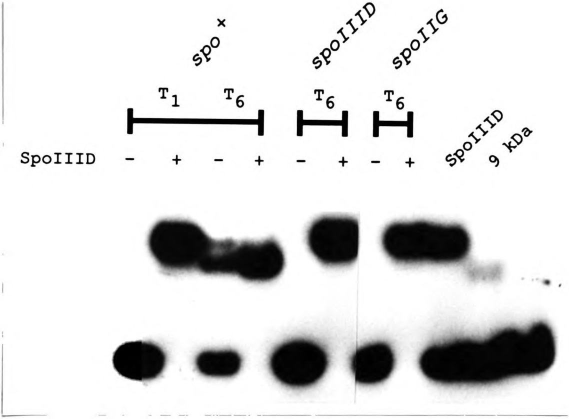
Figure 6. Mobility shift assay to monitor the levels of SpoIIID and the 9 kDa protein in wild-type and *spoIIID* mutant cells. A labelled oligonucleotide (50 ng) designed to correspond to a SpoIIID/9 kDa protein binding site in the *sigK* ORF (+73 to +96) was incubated with extracts (5 µg total protein) prepared from wild-type and *spoIIID* mutant cells that were collected at the indicated times after the onset of sporulation, gel-purified SpoIIID (30 ng) and gel-purified 9 kDa protein (5 ng), or gel-purified 9 kDa protein (30 ng). The mixtures were electrophoresed on a 20% polyacrylamide gel. (A) Extracts were prepared immediately before incubation with labelled DNA probe. (B) Extracts were incubated overnight at 4°C prior to incubation with the labelled DNA probe.


A
spo +
spoIIID
-
T1 T2 T3 T4 T5 T6 T7 T8
T1 T3 T5
SpoIIID + 9 kDa
9 kDa
B
spo +
-
T1 T2 T3 T4 T5 T6 T7 T8

kDa protein complex suggests that the 9 kDa protein never accumulates to a very high level in cells. Surprisingly, the relative amounts of the SpoIIID and 9 kDa protein complexes changed when the extracts were incubated overnight at 4°C (Figure 6B). The SpoIIID complex was only detected between T_3 and T_5. The 9 kDa protein complex was still detected between T_4 and T_8, but its level was significantly higher in this experiment as compared to the experiment performed with freshly prepared extracts (Figure 6A). A simple explanation for these results is that SpoIIID may be converted to the 9 kDa protein during the overnight incubation at 4°C. The presence of phenylmethylsulfonyl fluoride (PMSF, a serine protease inhibitor) in the extract buffer may stabilize the 9 kDa protein *in vitro*, allowing it to accumulate to a higher level than *in vivo*, because the levels of both SpoIIID and the 9 kDa protein were significantly reduced when PMSF was omitted from the extract buffer (data not shown).

Conversion of SpoIIID to the 9 kDa protein is reduced in several sporulation mutants. To test whether mutants that failed to produce SpoIIID were capable of converting SpoIIID to the 9 kDa protein, gel-purified SpoIIID was added to crude extracts prepared from these mutants, the mixture was incubated overnight at 4°C, and then the levels of SpoIIID and the 9 kDa protein were determined using the mobility shift assay (Figure 7). As a control, gel-purified SpoIIID was added to extracts prepared from wild-type cells.

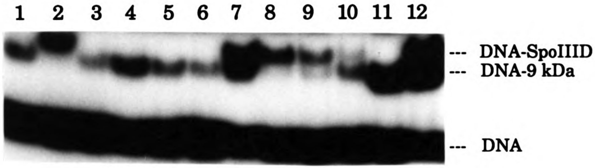
Figure 7. Mobility shift assay to determine whether gel-purified SpoIIID is converted to the 9 kDa protein in extracts prepared from wild-type, *spoIIG* and *spoIIID* cells. Extracts (5 μg total protein) prepared from wild-type, *spoIIG* and *spoIIID* cells collected at the indicated times were incubated overnight at 4°C alone, or with gel-purified SpoIIID (15 ng). The extracts were then incubated with a labelled 106 bp *Eco*47III-*Hind*III fragment (100 ng) described in Materials and Methods. Gel-purified 9 kDa protein (15 ng; this particular preparation had low DNA-binding activity) and gel-purified SpoIIID (15 ng) were also incubated with the labelled DNA fragment. The mixtures were electrophoresed on an 8% polyacrylamide gel.


spo +
spoIIID
spoIIG
T1
T6
T6
T6
SpoIIID
- + - + - + - +
SpoIIID
9 kDa

Gel-purified SpoIIID failed to be converted to the 9 kDa protein in extracts prepared from wild-type cells harvested at T_1, but was converted in extracts prepared from wild-type cells harvested at T_6. Extracts prepared from *spoIIG* (encoding σ^E which is required for *spoIIID* transcription) and *spoIIID* mutant cells harvested at T_6 failed to convert gel-purified SpoIIID to the 9 kDa protein. These results demonstrate that the ability to convert SpoIIID to the 9 kDa form is a developmentally-regulated event that depends upon σ^E and SpoIIID.

To determine whether other sporulation genes affected the conversion, the levels of SpoIIID and the 9 kDa protein in sporulation mutants were monitored by mobility shift assays (Figure 8). Mutants that fail to produce pro-σ^K or σ^K (Lu *et al.*, 1990), due to a mutation in *sigK* (Stragier *et al.*, 1989) or a mutation in *spoIVCA* (encoding the putative recombinase that generates the composite *sigK* gene (Kunkel *et al.*, 1990; Sato *et al.*, 1990; Popham & Stragier, 1992)), produced the 9 kDa protein. Similarly, mutants that produce pro-σ^K but fail to produce a detectable amount of σ^K, due to a mutation in *spoIVB* or *spoIVF* (Lu *et al.*, 1990), produced the 9 kDa protein. These results demonstrate that the conversion does not depend on σ^K. In fact, the level of the 9 kDa

Figure 8. Mobility shift assay to monitor the levels of SpoIIID and the 9 kDa protein in sporulation mutants. A labelled 106 bp *Eco*47III-*Hind*III fragment (100 ng) described in Materials and Methods was incubated with extracts (2 μg) prepared from wild-type or mutant cells that were collected at T_6, gel-purified 9 kDa protein (30 ng; lane 11), or a combination (lane 12) of gel-purified SpoIIID (30 ng) and 9 kDa protein (30 ng). These mixtures were electrophoresed on an 8% polyacrylamide. Note: Extracts were incubated overnight at 4°C prior to incubation with the labelled DNA fragment. Lanes: 1, PY79 (spo^+), 2, BK183 (*spoIVA*), 3, (*spoIVB*), 4, BK558 (*spoIVCA*), 5, BK103 (*spoIVCB*), BSL51 (*spoIVF*), 91 (*spoVB*), SC50 (*spoVC*), SC53 (*spoVF*), and BZ216 (*cotE*).


1
2
3
4
5
6
7
8
9
10
11
12
--- DNA-SpoIIID
--- DNA-9 kDa
--- DNA

protein is significantly higher in fresh extracts prepared from mutants that fail to produce σ^K, as compared to wild-type cells (data not shown), suggesting that active σ^K may interfere with the conversion and/or cause destabilization of the 9 kDa protein. Mutants that produce abnormal spores with altered cortex and/or coat layers (Piggot *et al.*, 1981) either fail to convert SpoIIID to the 9 kDa protein (*spoIVA*) or are impaired in the conversion (*spoVB*, *spoVC* and *spoVF*). These observations suggest that the conversion of SpoIIID to the 9 kDa protein is coupled to spore morphogenesis.

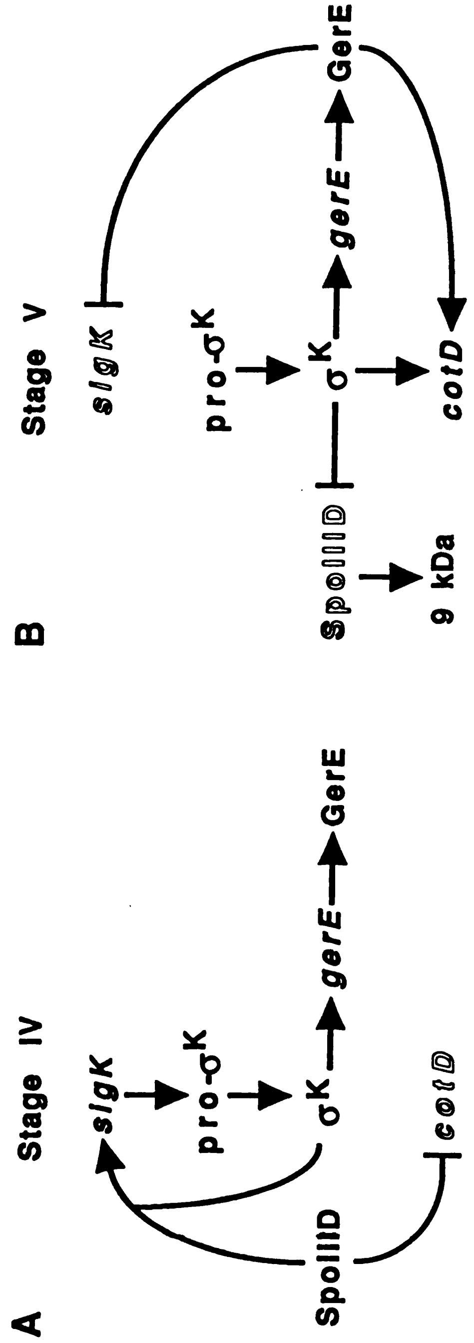
Discussion

The results presented here provide evidence that SpoIIID is converted to a 9 kDa form by removing 7 amino acids from its C-terminus. While this conversion did not alter the ability of the protein to bind to specific sites in DNA (Figure 4), it did appear to alter the ability of the protein to affect transcription (Figure 5). The conversion is developmentally regulated, since (1) SpoIIID present at T_3 was not converted to the 9 kDa protein during sporulation (Figure 6) and (2) the conversion is defective in several sporulation mutants (Figures 7 and 8). The 9 kDa protein does not appear to accumulate to a very high level in cells, suggesting that it is unstable. Hence, conversion of SpoIIID to the 9 kDa form may explain the parallel decrease in the levels of *spoIIID* mRNA and SpoIIID protein during sporulation (Halberg & Kroos, 1992). Because SpoIIID influences the transcription of many mother-cell genes (Kroos *et al.*, 1989; Zheng *et al.*, 1992), the change in its transcriptional effects and cellular stability brought about by conversion to the 9 kDa form presumably has a profound effect on the pattern of mother-cell gene expression.

How might the SpoIIID protein be converted to the 9 kDa form? The total amount of SpoIIID and the 9 kDa protein is comaparable in fresh and overnight extracts, but the relative amounts of SpoIIID and the 9 kDa protein are different in these extracts (i.e., the amount of SpoIIID is lower and the amount of the 9 kDa protein is higher in overnight extracts as compared to fresh extracts; Figure 6). This observation suggests that SpoIIID is converted to a 9 kDa form. A simple explanation for the conversion is proteolytic processing involving either a single endoproteolytic cleavage or several exoproteolytic cleavages. In these cases, the convertase (i.e., the factor that catalyzes the conversion) would be a protein. Another possible explanation is that SpoIIID may autoproteolyze. In this case, the convertase may or may not be a protein.

Alternatively, SpoIIID may not be converted to a 9 kDa form. The 9 kDa protein may be generated by translational frameshifting. If this is the case, translation would have to occur *in vitro*, since the amount of the 9 kDa protein in the extracts increases during the overnight incubation (Figure 6). The frameshift would have to occur downstream of the valine at position 20, because the first 20 amino acids of the 9 kDa protein is identical to those of SpoIIID. In addition, it would most likely occur downstream of the threonine at position 42, because the 9 kDa protein and SpoIIID have identical binding sites in two genes (Figure 5) and the putative helix turn helix DNA-binding motif of SpoIIID is located between positions 23-42 (Kunkel *et al.*, 1989). Translational frameshifting often requires two sequence elements: a slippery site, which is composed of

Figure 9. Model for the switch from *sigK* to *cotD* transcription in the mother cell during the stage IV to stage V transition of sporulation. (A) During stage IV, SpoIIID stimulates *sigK* and represses *cotD* transcription by σ^K RNA polymerase. σ^K RNA polymerase also transcribes *gerE*, leading to the synthesis of GerE. (B) The accumulation of σ^K, resulting from the processing of pro-σ^K to σ^K, causes a decrease in the level of *spoIIID* mRNA. This decrease is paralleled by a decrease in the level of SpoIIID, which appears to be due to the conversion of SpoIIID to a less stable 9 kDa form. The SpoIIID decrease causes a switch to the stage V pattern of gene expression (i.e., *sigK* transcription is no longer stimulated and *cotD* transcription is no longer repressed). Continued production of GerE reinforces the switch since GerE represses *sigK* transcription and stimulates *cotD* transcription.


A
Stage IV
slgK
pro-σK
σK
gerE
GerE
SpoIIID
cotD
B
Stage V
slgK
pro-σK
σK
gerE
GerE
SpoIIID
9 kDa
cotD

stretch of adenosines, and a stem-loop structure. A potential slippery site exists in the C-terminus of the spoIIID gene and a -1 shift would result in a premature stop. However, there is no stem-loop structure and the predicted mass of the polypeptide produced by such a frameshift is smaller than the observed mass of the 9 kDa protein.

What role could the conversion of SpoIIID to the 9 kDa form play in establishing the temporal pattern of mother-cell-specific gene expression? SpoIIID activates *sigK* transcription and represses *cotC*, *cotD*, and *cotX* transcription by σ^K RNA polymerase *in vitro* (H. Ichikawa, R. Halberg, L. Kroos, unpublished data; H. Ichikawa and L. Kroos, unpublished data; Kroos *et al.*, 1989). Previously, it was proposed that the inactivation and/or sequestering of SpoIIID switches the mother-cell pattern of gene expression from the transcription of *sigK* during stage IV to the transcription of spore coat genes during stage V (Kroos & Losick, 1989). Consistent with this idea, the accumulation of σ^K, resulting from the processing of pro-σ^K to σ^K, reduces the levels of *spoIIID* mRNA and SpoIIID (Halberg & Kroos, 1992). The reduction in the level of *spoIIID* mRNA would not be concomitant with a reduction in the level of SpoIIID protein unless there was a mechanism to inactivate the SpoIIID protein. Thus, the conversion of SpoIIID to the 9 kDa protein may be required to relieve the repressive effect that SpoIIID has on the expression of spore coat proteins during sporulation (Figure 9). Protein stability plays a role in controlling the pattern of gene expression in other biological systems. For example, the stability of the cII protein is a key element in the lysogeny-lysis decision of bacteriophage λ (i.e., the presence of cII favors lysogeny (Echols & Green, 1971; Reichardt & Kaiser, 1971; Shimatake & Rosenberg, 1981; Ho *et al.*, 1983; McMacken *et al.*, 1970), whereas the absence of cII, resulting from its degradation by host factors (Hoyt *et al.*, 1982; Rattray *et al.*, 1984; Banuett *et al.*, 1986; Cheng *et al.*, 1988), favors lysis).

How is the production of the convertase regulated during sporulation? The production of convertase depends on σ^E and SpoIIID (Figure 7). Perhaps σ^E RNA polymerase transcribes a gene required to produce convertase and this transcription may be stimulated by SpoIIID. SpoIIID does activate and repress the transcription of several σ^E-dependent genes (Errington, 1993; Chapter II). Errington (1993) proposed that the transcriptional effects of SpoIIID may divide the σ^E regulon into three temporal classes. The first class is composed of genes whose transcription is unaffected by SpoIIID. The second class is composed of genes whose expression is repressed by SpoIIID. Expression of class I and II genes would begin as soon as σ^E activity appears. The third class is composed of genes whose expression is stimulated by SpoIIID. Expression of class III genes would be delayed relatively to classes 1 and 2, because transcription of the *spoIIID* gene by σ^E RNA polymerase is itself partially dependent on SpoIIID (Kunkel *et al.*, 1989). Thus, a gene required to produce convertase may belong to temporal class III of the σ^E regulon.

Alternatively or in addition, perhaps σ^E and SpoIIID are required indirectly to permit synthesis and/or activation of the convertase. No matter whether SpoIIID affects convertase directly or indirectly, a lag between the appearance of SpoIIID and appearance of the 9 kDa protein would be expected, and a lag of about one hour is observed (Figure 6). Conversion is inhibited by mutations in two genes that are known to be σ^E dependent, *spoIVA* (Roels *et al.*, 1992) and *spoVB* (Popham & Stragier, 1991). Interestingly, mutations in two of these genes, *spoIVA* and *spoVB*, impair formation of the spore cortex (Roels *et al.*, 1992; Popham & Stragier, 1991). Thus, cortex formation may be required for full convertase activity. An appealing feature of this model is that the relief of the SpoIIID repressive effect on the transcription of spore coat genes is coupled to the morphological event preceding the formation of the spore coat. However, mutations that block σ^K production also inhibit cortex formation, yet SpoIIID is converted to the 9 kDa protein. One possible explanation for this observation is that the convertase does not gain full activity, but convertase accumulates due to the sporulation block caused by the absence of σ^K. Consistent with the former is the finding that a mutation in *spoVF*, a locus whose transcription depends on σ^K RNA polymerase, inhibits the conversion (Figure 8). Consistent with the idea that convertase accumulates due to the absence of σ^K is the observation that the level of SpoIIID is higher in mutants that fail to produce σ^K than in wild-type cells (Halberg & Kroos, 1992), which demonstrates that σ^K can affect the accumulation of a protein whose expression depends on σ^E.

The *spoVF* operon is composed of two genes, *dpaA* and *dpaB*, which encode subunits of dipicolinic acid (DPA) synthetase (Daniel & Errington, 1993). DPA synthetase is required for the formation of DPA from an intermediate in the lysine biosynthetic pathway (Bach & Gilvarg, 1966). DPA is a small, polar molecule that appears to play a role in spore heat-resistance (Church & Halvorson, 1959). Hence, conversion of SpoIIID to the 9 kDa protein may couple release of spore coat repression to acquisition of heat-resistance as well as formation of the spore cortex.

CHAPTER V

Overproduction of SpoIIID Negatively Regulates σ^K Accumulation During *Bacillus subtilis* Sporulation and Reduces *cotD* Expression in Cells Producing σ^K During Growth

"In everything the middle course is the best; all things in excess bring trouble."

Plautus

Abstract

SpoIIID is a DNA-binding protein that activates or represses transcription of many different genes in the mother-cell compartment of sporulating *Bacillus subtilis*. Previous studies showed that SpoIIID represses *cotD* (encoding a spore coat protein) transcription by σ^K RNA polymerase *in vitro* and that a decrease in the level of SpoIIID coincides with an increase in *cotD* expression during sporulation. Hence, SpoIIID was proposed to repress *cotD* transcription *in vivo* until the level of SpoIIID falls. We attempted to test this hypothesis by engineering continued production of SpoIIID late in sporulation. We found that elevating the level of SpoIIID reduced the expression of all σ^K-transcribed genes tested, including some not expected to be repressed by SpoIIID. The production of heat-resistant spores was also reduced. These effects appear to result from a reduction in the level of σ^K. Thus, maintaining the proper levels of SpoIIID and σ^K is crucial for normal sporulation. To circumvent the problem of SpoIIID overproduction reducing the level of σ^K during sporulation, we employed a strain engineered to produce σ^K during growth. Expressing SpoIIID in this strain during growth reduced *cotD* expression, but not the expression of other σ^K-transcribed genes tested. These

results suggest that SpoIIID is capable of repressing *cotD* expression *in vivo*.

Introduction

During sporulation of *Bacillus subtilis,* an asymmetrically positioned septum divides the bacterium into two compartments, the mother cell and the forespore (Smith *et al.,* 1989). Both of these compartments receive a copy of the genome, but realize alternative developmental fates because gene expression is regulated spatially. Two key regulators of gene expression in the mother-cell compartment are SpoIIID, a small DNA-binding protein that functions as a transcriptional activator and repressor (Chapter II; Kroos *et al.,* 1989; Kunkel *et al.,* 1989), and σ^K, a sigma subunit of RNA polymerase (Kroos *et al.,* 1989; Stragier *et al.,* 1989; Zheng & Losick, 1990; Zheng *et al.,* 1992).

Production of σ^K is regulated positively by SpoIIID at several levels. First, SpoIIID is required for a chromosomal rearrangement that generates the composite *sigK* gene encoding σ^K (Stragier *et al.,* 1989; Kunkel *et al.,* 1990). Second, SpoIIID stimulates *sigK* transcription (Kunkel *et al.,* 1988; Kroos *et al.,* 1989). Third, SpoIIID is needed to permit proteolytic processing of the *sigK* primary translation product, pro-σ^K, to produce active σ^K (S. Lu & L. Kroos, unpublished data). Several lines of evidence indicate that processing of pro-σ^K couples events occurring in the mother cell to events occurring in the forespore (Cutting *et al.*, 1990; Lu *et al.*, 1990; Cutting *et al.*, 1991a; Cutting *et al.*, 1991b).

In addition to positively regulating σ^K production, SpoIIID represses *cotD* (encoding a spore coat protein (Donovan *et al.*, 1987)) transcription by σ^K RNA polymerase *in vitro* (Kroos *et al.*, 1989). Hence, it was proposed that inactivation of SpoIIID switches the pattern of mother-cell gene expression from *sigK* transcription at stage IV (cortex formation) of sporulation to *cotD* transcription at stage V (coat formation) (Kroos *et al.*, 1989). Previously, we demonstrated that the level of SpoIIID does decrease during sporulation and that the decrease is controlled, at least in part, by the production of σ^K (Halberg & Kroos, 1992). We also noted that the SpoIIID decrease coincides with an increase in *cotD* expression during sporulation in wild-type cells and cells engineered to produce σ^K earlier than normal, suggesting that SpoIIID represses *cotD* expression *in vivo* (Halberg & Kroos, 1992).

Here we attempted to test the idea that SpoIIID represses *cotD* expression *in vivo* by engineering continued production of SpoIIID late in sporulation. Unexpectedly, we found that elevating the level of SpoIIID not only reduced *cotD* expression, but the expression of all σ^K-transcribed genes tested and the production of heat-resistant spores. We show that overproducing SpoIIID reduces σ^K accumulation during sporulation. To circumvent this problem, we employed a strain that was engineered to permit the production of σ^K during vegetative growth. We show that expression of SpoIIID in this strain reduces *cotD* promoter activity, but not the promoter activity of other σ^K-transcribed genes, suggesting that SpoIIID is capable of repressing *cotD* expression *in vivo*.

Materials and Methods

Bacterial Strains

E. coli strain AG115 (*araD139*Δ(*ara,leu*)*7697*, Δ*lacX74*, *galU-*, *galK-*, *hsr-*, *hsm*$^+$, *strA*, (F', *proAB*, *lacI*^{q}Z::Tn5)) was obtained from A. Grossman (Massachusetts Institute of Technology) and served as the host for construction and maintenance of plasmids. *B. subtilis* strains PY79 (*spo*$^+$), BK395 (*spoIIID83*), BK541 (*spoIIID*Δ*erm*), and VO536 (P*spac*-P*sigK*-*sigK*Δ*19*) have been described (Youngman *et al.*, 1984; Kunkel *et al.*, 1989; Oke & Losick, 1993).

P*spac-spoIIID* was derived from pBK39 (Kunkel *et al.*, 1989) which contains *spoIIID*, and pDG148 (Stragier *et al.*, 1988) which contains the isopropyl ß-D-thiogalactopyranoside (IPTG)-inducible promoter, *spac*, and is stably maintained in *E. coli* or *B. subtilis*. pDG148 was digested with *Hind*III. The ends were rendered blunt by the fill-in reaction of Klenow enzyme and then dephosphorylated with calf intestinal phosphatase. The linearized plasmid was ligated to a 536 bp *Xmn*I fragment from pBK39 containing the *spoIIID* promoter region and open reading frame (ORF). Ampicillin-resistant *E. coli* transformants were obtained and the structure of P*spac-spoIIID*, a plasmid containing the insert in the proper orientation to fuse *spoIIID* transcription to *spac*, was verified by restriction mapping.

P*spac-spoIIIDΔ* is identical to P*spac-spoIIID* except it lacks the *spoIIID* ORF. It was generated following the procedure outlined above except the insert was a 234 bp *Xmn*I-*Apa*LI fragment from pBK39 containing only the *spoIIID* promoter region. The *Apa*LI end was rendered blunt using the fill-in reaction of Klenow enzyme prior to ligation.

P*spac-spoIIID-cat* is an integrational version of P*spac-spoIIID*. It was constructed by replacing the *Eco*RI fragment encoding kanamycin resistance and the origin of replication that functions in *B. subtilis* with a chloramphenicol resistance-encoding *Eco*RI fragment from pMI1101 (Igo & Losick, 1986).

Competent *B. subtilis* cells were prepared and transformed as described (Dubnau & Davidoff-Abelson, 1971). Transformants containing P*spac-spoIIID* and P*spac-spoIIIDΔ* were selected on LB agar containing kanamycin sulfate (5 mg/ml). Transformants containing P*spac-spoIIID-cat* integrated into the chromosome were selected on LB agar containing chloramphenicol (5 µg/ml).

Use of specialized transducing phages SPß::*cotA-lacZ*, SPß::*cotD-lacZ*, SPß::*gerE-lacZ*, and SPß::*sigK-lacZ* has been described (Kunkel *et al.*, 1988; Cutting *et al.*, 1989; Cutting *et al.*, 1990; Zheng & Losick, 1990).

Growth and Sporulation

Sporulation was induced by resuspending growing cells in SM medium as described (Sterlini & Mandelstam, 1969). The onset of sporulation (T_0) is defined as the time of resuspension. At three hours after sporulation, IPTG was added to a final concentration of 1 mM. Production of heat-resistant spores was assayed as described (Cutting & Horn, 1990).

Pspac-P*sigK-sigKΔ19* cells were grown in 2 x YT medium (Maniatis *et al.*, 1982). When cells reached the mid-log phase (O.D.$_{595}$ of 0.3-0.5), IPTG was added to a final concentration of 1 mM.

Western blot analysis

Samples (1 ml) were harvested by centrifugation (14,000 *g* for 5 minutes) at the indicated times during growth and sporulation. Whole-cell extracts were prepared as described (Halberg & Kroos, 1992) and the amount of protein present was quantified by the Bradford method (Bradford, 1976). Polypeptides (5 μg) were separated by SDS-PAGE (18% polyacrylamide; Thomas & Kornberg, 1978) and electroblotted to poly(vinylidene difluoride) membrane (Matsudaira, 1987). Immunoblot analyses using polyclonal anti-SpoIIID and anti-pro-σ^K antibodies were performed as described (Halberg & Kroos, 1992).

ß-galactosidase assays

ß-galactosidase activity was determined using the substrate *o*-nitrophenol-ß-D-galactoside (ONPG) as described (Miller, 1972). One unit of enzyme hydrolyzes 1 µmol of substrate per min per O.D.$_{595}$ of initial cell density.

In vitro transcription assays

In vitro transcription assays were performed utilizing σ^K RNA polymerase which was partially purified from *gerE*- cells and SpoIIID which was gel-purified as described (Kroos *et al.*, 1989).

Northern blot analysis

At hourly intervals between T_4 and T_8, cells were harvested by centrifugation (11,950*g* for 10 minutes) and RNA was prepared as described (Halberg & Kroos, 1992). The RNA was treated with DNase I to remove contaminating chromosomal DNA. The RNA (20 µg) was fractionated by electrophoresis on a 1.2% (w/v) agarose gel containing 1.11% (v/v) formaldehyde, transferred to nitrocellulose, and hybridized at 55°C to nick-translated pSC146 (Zheng *et al.*, 1992) or pLRK100 (Kroos *et al.*, 1989). The signals were visualized by autoradiography and quantitated using a Visage Digital Imager. The size of the mRNAs was estimated by comparing the positions of the signals to the positions of RNA standards

(0.16 kb to 1.77 kb RNA ladder from BRL).

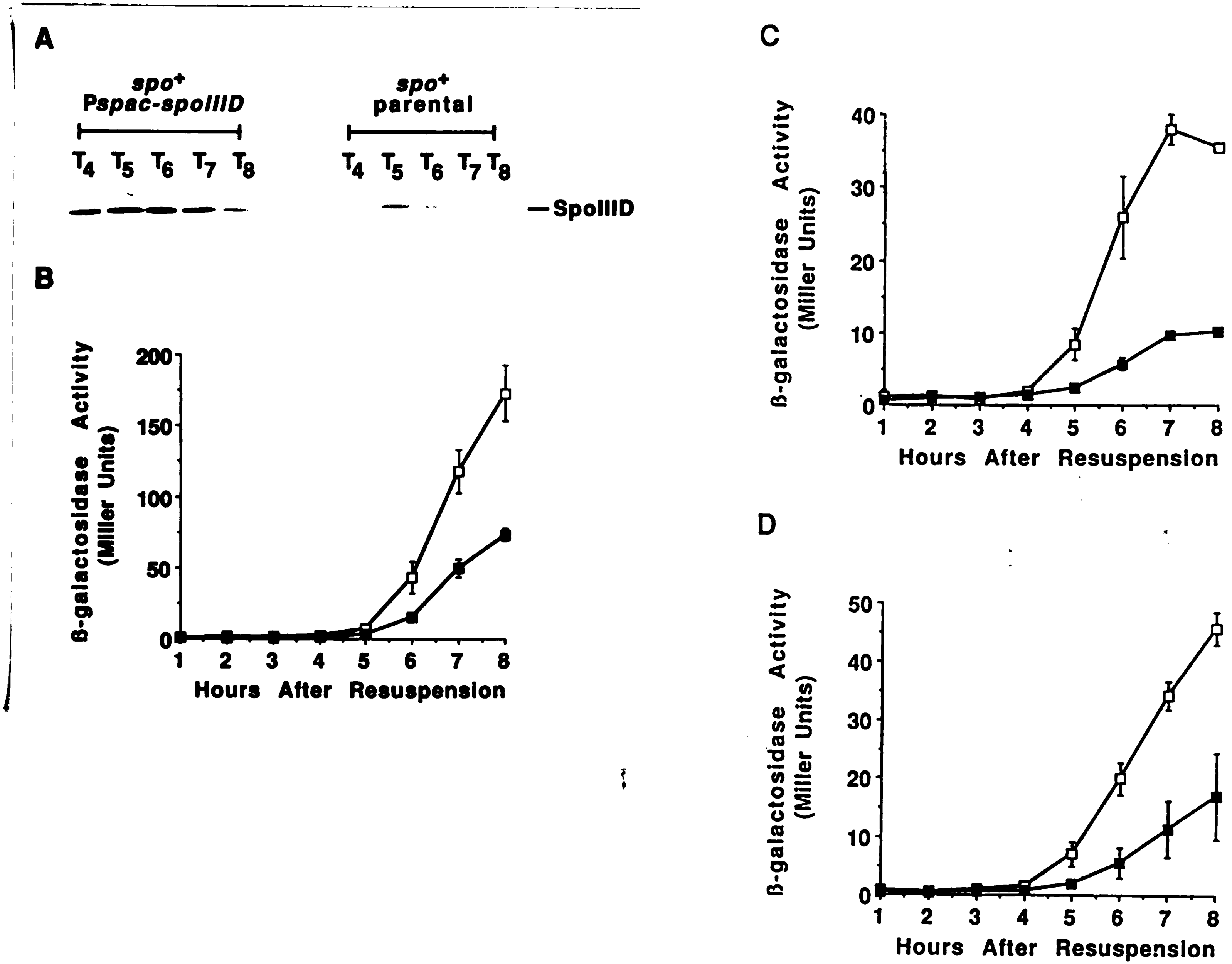
Results

A plasmid containing the *spoIIID* gene permits continued production of SpoIIID late in sporulation. To produce SpoIIID, a multicopy plasmid (P*spac-spoIIID*) was constructed containing the IPTG-inducible *spac* promoter fused to the *spoIIID* promoter region and ORF (see Materials and Methods). P*spac-spoIIID* and its parental plasmid pDG148 (Stragier *et al.*, 1988) were transformed into *spoIIID83* cells which fail to produce SpoIIID (R. Halberg and L. Kroos, unpublished data). Sporulation was induced in the resulting strains by the resuspension method and IPTG was added at T_3 (i.e., three hours after sporulation, which is the time when SpoIIID is first detected in wild-type cells (Halberg & Kroos, 1992)). The level of SpoIIID was monitored by Western blot analysis utilizing polyclonal antiserum against SpoIIID. *spoIIID-* cells containing P*spac-spoIIID* accumulated more SpoIIID than wild-type (*spo*$^+$) cells, whereas *spoIIID-* cells containing the parental plasmid failed to produce SpoIIID (data not shown). P*spac-spoIIID* not only elevated the amount of SpoIIID present between T_3 and T_5, but permitted a significant level of SpoIIID to be maintained between T_6 and T_8, when the level of SpoIIID is normally very low (Halberg & Kroos, 1992). Interestingly, the level of SpoIIID was also elevated in P*spac-spoIIID*-containing cells that were not treated with IPTG (data not shown). This result suggests that overproduction of SpoIIID is due to the presence of multiple copies of the *spoIIID* gene (including the promoter) and/or incomplete repression of the *spac* promoter. Nonetheless, the goal of elevating the level of SpoIIID late in sporulation was achieved.

Overproduction of SpoIIID reduces *cotD*, *cotA*, and *gerE* promoter activity. If SpoIIID does repress *cotD* transcription *in vivo*, then an elevated level of SpoIIID should reduce *cotD* expression. To test this prediction, a *cotD-lacZ* fusion was introduced into wild-type cells containing either P*spac-spoIIID* or the parental plasmid. Sporulation was induced in the resulting strains by the resuspension method and IPTG was added at T_3. The levels of SpoIIID and *cotD*-directed ß-galactosidase activity were monitored. Cells containing P*spac-spoIIID* exhibited an elevated level of SpoIIID between T_4 and T_8 relative to that observed in cells containing the parental plasmid (Figure 1A). Overproduction of SpoIIID reduced *cotD* expression two-fold (Figure 1B). These results are consistent with the hypothesis that SpoIIID represses *cotD* expression *in vivo*.

However, overproduction of SpoIIID also reduced the expression of two other σ^K-transcribed genes, including one not expected to be repressed by SpoIIID. Preliminary studies indicated that SpoIIID represses *cotA* (encoding a spore coat protein (Donovan *et al.*, 1987)) transcription by σ^K RNA

Figure 1. Levels of SpoIIID and *cotD-*, *cotA-* , and *gerE*-directed ß-galactosidase activity in *spo*$^+$ cells containing either P*spac-spoIIID* or the parental plasmid. The level of SpoIIID (panel A) in *spo*$^+$ cells containing P*spac-spoIIID* or the parental plasmid (pDG148), collected between T_4 and T_8 was determined by Western blot analysis as described in the Materials and Methods. *cotD-*, *cotA-*, and *gerE*-directed ß-galactosidase activity (panels B, C, and D, respectively) detected in *spo*$^+$ cells containing either P*spac-spoIIID* (■) or the parental plasmid, pDG148 (□). Points are the average of three determinations for *cotD*-directed ß-galactosidase activity and the average of two determinations for *cotA-* and *gerE*-directed ß-galactosidase activity. Error bars indicate one standard deviation of the data.

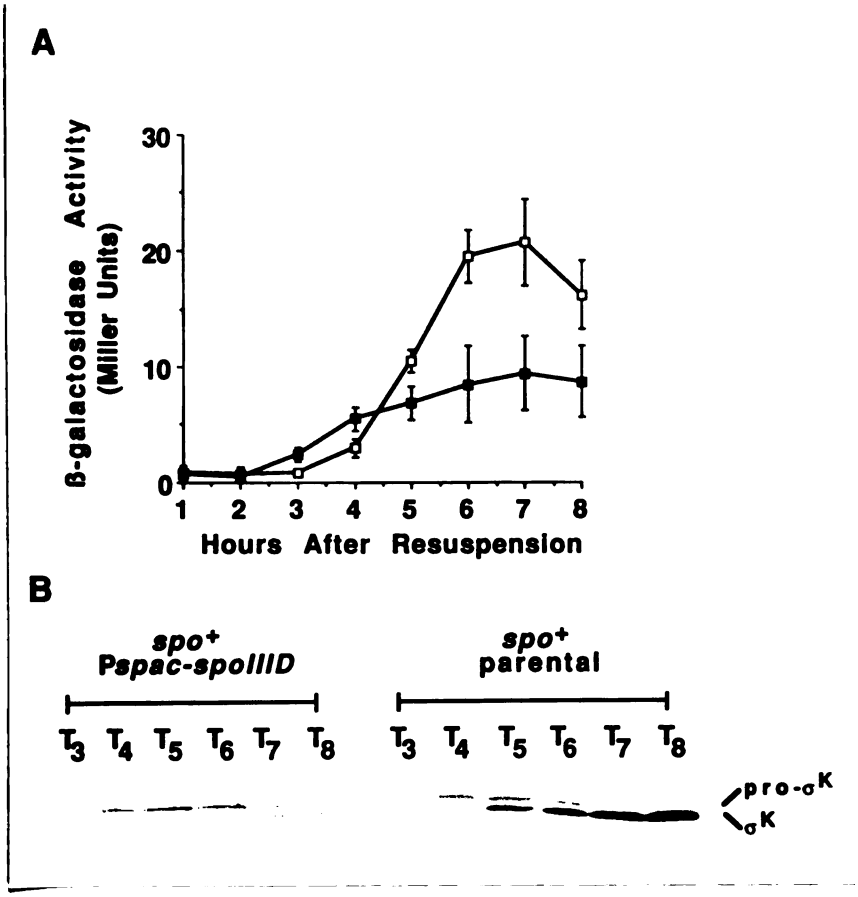

A
spo+
Pspac-spoIIID
T4 T5 T6 T7 T8
spo+
parental
T4 T5 T6 T7 T8
—SpoIIID
B
ß-galactosidase Activity (Miller Units)
0
50
100
150
200
1 2 3 4 5 6 7 8
Hours After Resuspension
C
ß-galactosidase Activity (Miller Units)
0
10
20
30
40
1 2 3 4 5 6 7 8
Hours After Resuspension
D
ß-galactosidase Activity (Miller Units)
0
10
20
30
40
50
1 2 3 4 5 6 7 8
Hours After Resuspension

polymerase *in vitro*, but has no effect on *gerE* (encoding a regulator of spore coat synthesis (Cutting & Mandelstam, 1986)) transcription by σ^K RNA polymerase *in vitro* (L. Kroos & R. Losick, unpublished data). Based on these observations, overproduction of SpoIIID was expected to reduce *cotA* promoter activity, but have no effect on *gerE* promoter activity. To test this prediction, *lacZ* fusions to *cotA* and *gerE* were introduced into wild-type cells containing either P*spac-spoIIID* or the parental plasmid. Sporulation was induced in the resulting strains by the resuspension method and IPTG was added at T_3. *cotA*-directed ß-galactosidase activity was reduced four-fold (Figure 1C) and *gerE*-directed ß-galactosidase activity was reduced three-fold (Figure 1D) in cells containing P*spac-spoIIID* as compared to cells containing the parental plasmid. The unexpected reduction in *gerE-lacZ* expression suggests either that SpoIIID does repress *gerE* expression *in vivo* or that the overproduction of SpoIIID indirectly affects the expression of all σ^K-dependent genes by negatively regulating the level of σ^K or its activity.

Overproduction of SpoIIID reduces *sigK* promoter activity and the level of σ^K. Since SpoIIID greatly activates *sigK* transcription by σ^E RNA polymerase (Chapter II) and σ^K RNA polymerase (Kunkel *et al.*, 1988; Kroos *et al.*, 1989), overproduction of SpoIIID was expected to have a positive effect on *sigK* promoter activity. To test this prediction, wild-type cells containing either P*spac-spoIIID* or the parental plasmid were transduced with phage carrying a *sigK-lacZ* fusion. Sporulation was induced in the resulting strains by the resuspension method and IPTG was added at T_3. *sigK*-directed ß-galactosidase activity was elevated slightly during the early stages of sporulation and reduced significantly during intermediate to late stages of sporulation in cells containing P*spac-spoIIID* as compared to cells containing the parental plasmid (Figure 2A). Elevated ß-galactosidase activity at T_3 and T_4 may reflect SpoIIID stimulating *sigK* transcription by σ^E RNA polymerase. The reduced ß-galactosidase activity at T_5 through T_8 may reflect a reduction in *sigK* transcription by σ^K RNA polymerase.

To determine whether the level of σ^K was lower in cells containing P*spac-spoIIID*, we used anti-pro-σ^K antibodies (Lu *et al.*, 1990) to detect pro-σ^K and σ^K in Western blot analyses (Figure 2B). The level of pro-σ^K was similar in strains containing P*spac-spoIIID* or the parental plasmid, suggesting that overproduction of SpoIIID does not hinder the chromosomal rearrangement that generates the composite *sigK* gene or its initial expression. However, the level of σ^K was

Figure 2. Levels of *sigK*-directed ß-galactosidase activity, pro-σ^K, and σ^K in *spo*$^+$ cells containing either P*spac-spoIIID* or the parental plasmid. (Panel A) *sigK*-directed ß-galactosidase activity detected in *spo*$^+$ cells containing either P*spac-spoIIID* (■) or the parental plasmid, pDG148 (□). Points are the average of three determinations. Error bars indicate one standard deviation of the data. (Panel B) The levels of pro-σ^K and σ^K in *spo*$^+$ cells containing either P*spac-spoIIID* or the parental plasmid (pDG148), collected between T_3 and T_8 were determined by Western blot analyses utilizing anti-pro-σ^K antibodies as described in the Materials and Methods.


A
ß-galactosidase Activity
(Miller Units)
30
20
10
0
1
2
3
4
5
6
7
8
Hours After Resuspension
B
spo+
Pspac-spoIIID
spo+
parental
T3
T4
T5
T6
T7
T8
T3
T4
T5
T6
T7
T8
pro-σK
σK

lower at T_4 through T_8 in cells containing P*spac-spoIIID* as compared to cells containing the parental plasmid. This shows that the overproduction of SpoIIID adversely affects the accumulation of σ^K. The lower level of σ^K may reduce expression of σ^K-dependent genes.

Because the level of σ^K, but not the level of pro-σ^K, was reduced by SpoIIID overproduction, we reasoned that processing of pro-σ^K to σ^K might be the step in σ^K production affected by elevating the level of SpoIIID. Since *bof* (bypass of the forespore) mutations bypass many of the normal requirements for pro-σ^K processing (Cutting *et al*., 1990), we examined expression of *cotD-*, *cotA-*, *gerE-* and *sigK*-lacZ fusions in a *bofB8* mutant containing either P*spac-spoIIID* or the parental plasmid. The results were similar to those observed in wild-type cells (data not shown). Hence, a *bof* mutation did not overcome the negative effect of SpoIIID overproduction on expression of σ^K-dependent genes.

Overproduction of SpoIIID inhibits the production of heat-resistant spores. The presence of P*spac-spoIIID* in *spoIIID-* cells only partially restored the production of heat-resistant spores (Table 1). Interestingly, the presence of P*spac-spoIIID* in wild-type cells reduced the production of heat-resistant spores to a number that was comparable to the number of heat-resistant spores produced in *spoIIID-* cells

Table 1. Number of heat-resistant spores produced by *Bacillus subtilis* strains in the absence and presence of SpoIIID overproduction. The number of heat-resistant spores produced by cells containing P*spac-spoIIID* or the parental plasmid (pDG148) was determined as described in the Materials and Methods. The values represent the number of heat-resistant spores per ml at T_{20} divided by the $O.D._{595}$ at T_1 and are the average of at least two independent determinations. The values in parentheses are the percentages of the number of heat-resistant spores produced in spo^+ cells containing the parental plasmid.

Strain	Heat-Resistant Spores
spoIIID83, parental	1.3×10^3 (4.6×10^{-4})
spoIIID83, P*spac-spoIIID*	1.9×10^7 (6.8)
spo$^+$, parental	2.8×10^8 (100)
spo$^+$, P*spac-spoIIID*	3.2×10^7 (11.4)
spo$^+$, P*spac-spoIIID*Δ	4.4×10^8 (157)

containing P*spac-spoIIID* (Table 1). One interpretation of these observations is that overproduction of SpoIIID and/or the resulting reduction in the level of σ^K inhibits sporulation.

An alternative interpretation is that the presence of multiple copies of the *spoIIID* promoter region might titrate a limiting factor that is important for sporulation. To test this possibility, a multicopy plasmid, P*spac-spoIIIDΔ*, was constructed (see Materials and Methods). This plasmid is identical to P*spac-spoIIID* except it lacks the entire *spoIIID* open reading frame. A *cotD-lacZ* fusion was introduced into wild-type cells containing P*spac-spoIIIDΔ*. Sporulation was induced in the resulting strain by the resuspension method and IPTG was added at T_3. Cells containing P*spac-spoIIIDΔ* exhibited normal levels of *cotD*-directed ß-galactosidase activity (data not shown) and heat-resistant spore production (Table 1). Thus, the presence of multiple copies of the *spoIIID* promoter region is not responsible for the effects of P*spac-spoIIID* on *cotD*-directed ß-galactosidase activity (Figure 1B) and heat-resistant spore production (Table 1).

Another interpretation of the effect of P*spac-spoIIID* on wild-type cells is that the copy of the *spoIIID* gene in P*spac-spoIIID* bears a mutation and its product exerts a dominant negative effect on sporulation. To test this possibility, the *spoIIID* gene in P*spac-spoIIID* was sequenced. No mutations were found (data not shown). Thus, overproduction of SpoIIID and/or the resulting reduction in the level of σ^K appears to be responsible for reducing *cotD* expression (Figure 1B) and inhibiting heat-resistant spore production (Table 1).

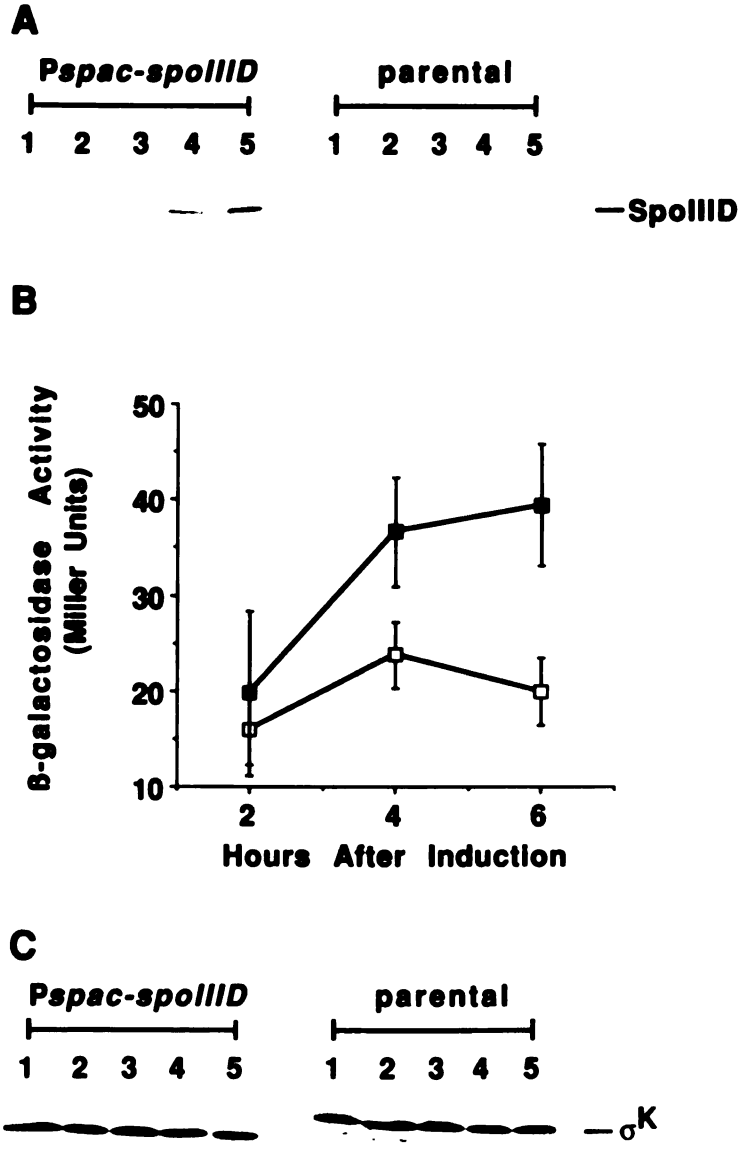
We attempted to alleviate the negative effect of the multicopy P*spac-spoIIID* plasmid on σ^K accumulation and spore production by integrating the P*spac-spoIIID* fusion into the bacterial chromosome at single copy. We constructed an integrational version of the plasmid, P*spac-spoIIID-cat* (see Materials and Methods), and allowed it to integrate into the chromosome of wild-type and *spoIIID*Δerm cells by single-reciprocal recombination. The resulting strains produced normal numbers of heat-resistant spores when IPTG was added at T_3 (data not shown). However, the level of SpoIIID as determined by Western blotting was not elevated late in sporulation (data not shown), but decreased as observed in wild-type cells ((Halberg & Kroos, 1992), Figure 1A). Therefore, integrating the P*spac-spoIIID* fusion into the chromosome did alleviate its negative effect on spore production, presumably by reducing the copy number, but the resulting strain was not useful for altering the level of SpoIIID late in sporulation.

Production of SpoIIID from P*spac-spoIIID* increases *sigK* expression and reduces *cotD* expression, but does not affect *cotA* and *gerE* expression, in cells

engineered to produce σ^K during vegetative growth.

Since overproduction of SpoIIID reduced the level of σ^K accumulated in cells during sporulation, a strain (P*spac*-P*sigK-sigK*Δ*19*) which contains the *spac* promoter fused to the *sigK* promoter and a truncated form of the *sigK* ORF (Oke & Losick, 1993) was employed to permit σ^K production during vegetative growth. The primary translation product of the truncated ORF is σ^K (with an additional methionine on its N-terminus) rather than pro-σ^K. By supplementing transcription of *sigK* with P*spac* and eliminating the need for processing to produce active σ^K, we hoped that overproduction of SpoIIID from P*spac-spoIIID* would not reduce the level of σ^K. We reasoned that expressing SpoIIID in this strain might actually increase the level of σ^K because SpoIIID stimulates *sigK* transcription by σ^K RNA polymerase (Kunkel *et al.*, 1988; Kroos *et al.*, 1989). To test this prediction, a *sigK-lacZ* fusion was introduced into P*spac*-P*sigK-sigK*Δ*19* cells containing either P*spac-spoIIID* or the parental plasmid. The resulting strains were grown in 2xYT medium and IPTG was added during the mid-log phase. The levels of SpoIIID, *sigK*-directed ß-galactosidase activity, and σ^K were monitored. Cells containing P*spac-spoIIID* produced SpoIIID, whereas

Figure 3. Levels of SpoIIID, *sigK*-directed ß-galactosidase activity, and σ^K in P*spac*-P*sigK-sigKΔ19* cells containing either P*spac-spoIIID* or the parental plasmid. (Panel **A**) The level of SpoIIID in P*spac*-P*sigK-sigKΔ19* cells containing P*spac-spoIIID* or the parental plasmid (pDG148), collected between 1 and 5 hours after IPTG induction was determined by Western blot analysis. (Panel B) *sigK*-directed ß-galactosidase activity detected in P*spac*-P*sigK-sigKΔ19* cells containing either P*spac-spoIIID* (■) or the parental plasmid, pDG148 (☐). Points are the average of three determinations. The background level of ß-galactosidase activity in cells not treated with IPTG was subtracted and did not exceed 2 units. Error bars indicate one standard deviation of the data. (Panel C) The level of σ^K in P*spac*-P*sigK-sigKΔ19* cells containing P*spac-spoIIID* or the parental plasmid (pDG148), collected between 1 and 5 hours after IPTG induction was determined by Western blot analysis.

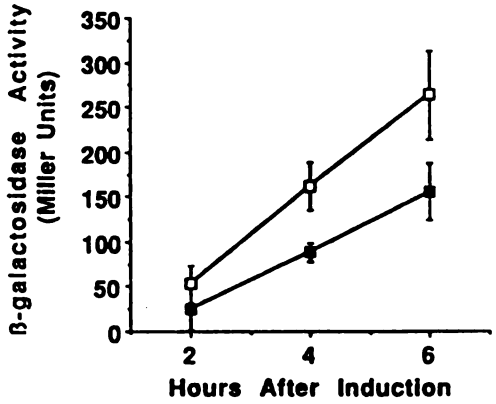

A
Pspac-spoIIID
1 2 3 4 5
parental
1 2 3 4 5
—SpoIIID
B
ß-galactosidase Activity (Miller Units)
50
40
30
20
10
2
4
6
Hours After Induction
C
Pspac-spoIIID
1 2 3 4 5
parental
1 2 3 4 5
—σK

cells containing the parental plasmid did not (Figure 3A). Producing SpoIIID in P*spac*-P*sigK*-*sigK*Δ*19* cells increased *sigK*-directed ß-galactosidase activity about two-fold between 4 and 6 hours after induction (Figure 3B), indicating that SpoIIID does stimulate *sigK* promoter activity *in vivo*. The level of σ^K in cells containing P*spac*-*spoIIID* is comparable to that observed in cells containing the parental plasmid (Figure 3C). Thus, these strains are suitable for testing the ability of SpoIIID to affect *cotD*, *cotA*, and *gerE* expression *in vivo*. *cotD*-, *cotA*- and *gerE*-*lacZ* fusions were introduced into P*spac*-P*sigK*-*sigK*Δ*19* cells containing either P*spac*-*spoIIID* or the parental plasmid. The resulting strains were grown in 2xYT medium and IPTG was added during the mid-log phase. The levels of *cotD*-, *cotA*- and *gerE*-directed ß-galactosidase activity were monitored. Producing SpoIIID reduced *cotD*-directed ß-galactosidase activity about two-fold between 4 and 6 hours after induction (Figure 4A), but had no effect on *cotA*- (Figure 4B) or *gerE*-directed ß-galactosidase activity (Figure 4C). These results suggest that SpoIIID is capable of repressing *cotD* expression *in vivo*, but provide no evidence for an effect of SpoIIID on *cotA* or *gerE* expression.

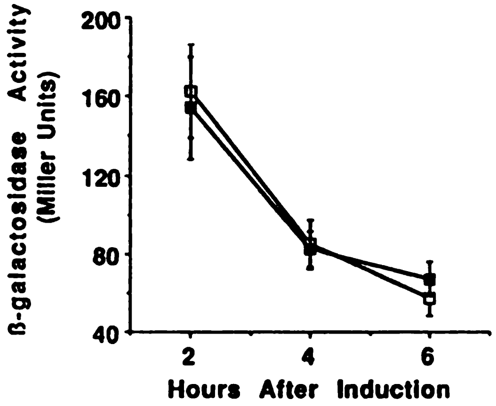
Additional evidence that *cotD*, but not *cotA* or *gerE*, is repressed by SpoIIID. The prediction that SpoIIID would repress *cotA*, but not *gerE*, expression *in vivo* was based on preliminary *in vitro* transcription studies (L. Kroos & R. Losick, unpublished data). The results shown in

Figure 4. Levels of *cotD-*, *cotA-* and *gerE*-directed ß-galactosidase activity in P*spac*-P*sigK-sigK*Δ*19* cells containing either P*spac-spoIIID* or the parental plasmid. *cotD-*, *cotA-* and *gerE*-directed ß-galactosidase activity (panels A, B and C, respectively) detected in P*spac*-P*sigK-sigK*Δ*19* cells containing either P*spac-spoIIID* (■) or the parental plasmid, pDG148 (□). Points are the average of three determinations for *cotD*-directed ß-galactosidase activity and the average of five determinations for *cotA-* and *gerE*-directed ß-galactosidase activity. The background level of ß-galactosidase activity observed in cells not treated with IPTG was subtracted and did not exceed 7, 5, or 25 units for *cotD-*, *cotA-* or *gerE-lacZ*, respectively. Error bars indicate one standard deviation of the data.

A


ß-galactosidase Activity
(Miller Units)
350
300
250
200
150
100
50
0
2
4
6
Hours After Induction

B


ß-galactosidase Activity
(Miller Units)
200
160
120
80
40
2
4
6
Hours After Induction

C

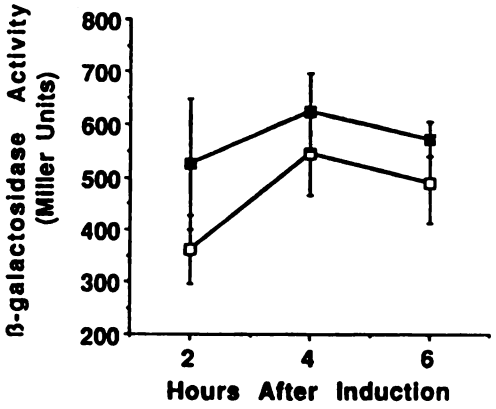

ß-galactosidase Activity
(Miller Units)
800
700
600
500
400
300
200
2
4
6
Hours After Induction

Figure 4B provided no evidence for a repressive effect of SpoIIID on *cotA* expression, so we repeated the *in vitro* transcription studies (Figure 5). SpoIIID markedly repressed *cotD* transcription and greatly stimulated *sigK* transcription (compare lanes 1 and 2), whereas it exhibited a slight repressive effect on *cotA* transcription (compare lanes 3 and 4) and *gerE* transcription (compare lanes 5 and 6). These results demonstrate that the ability of SpoIIID to repress *cotA* and *gerE* transcription is very weak relative to its ability to repress *cotD* transcription. A possible reason for the SpoIIID repression of *cotA* not appearing as strong in this study as compared to the previous one is that we used a different radioactively-labelled nucleotide (UTP), which provided a much stronger *in vitro* transcription signal.

Expression of *gerE* (Figure 1D) and *cotA* (Figure 1C) consistently began one hour earlier during sporulation than expression of *cotD* (Figure 1B) as measured by translational fusions to *lacZ*. A similar result was observed when the levels of *cotD* and *gerE* mRNA were measured in sporulating wild-type cells by Northern blot analysis (quantitatively summarized in Figure 6). The probes were pSC146 (Zheng *et al.*, 1992), which contains a 263 bp *Alu*I fragment of *B. subtilis* DNA spanning from 96 bp upstream of the *gerE* transcriptional start site to codon 35 of the *gerE* ORF, and pLRK100 (Kroos *et al.*, 1989), which contains a 430 bp *Eco*RI-*Hinc*II fragment of *B. subtilis* DNA spanning from 225 bp

Figure 5. Effects of SpoIIID on *cotD, sigK, cotA,* and *gerE* transcription *in vitro*. Linearized plasmid DNA (1 µg) was transcribed with partially purified σ^K RNA polymerase alone (0.2 µg), or with SpoIIID (0.24 µg) added immediately after the addition of RNA polymerase. Run-off transcripts were electrophoresed in 5% polyacrylamide gels containing 8 M urea and were detected by autoradiography. *cotD* transcription from *Hind*III-digested pLRK100 (225 base transcript) and *sigK* transcription from *Xba*I-digested pBK16 (170 base transcript) with σ^K RNA polymerase alone (lane 1) or with SpoIIID added (lane 2). *cotA* transcription from *Eco*RI-digested pKS23 (149 base transcript) with σ^K RNA polymerase alone (lane 3) or with SpoIIID added (lane 4). *gerE* transcription from *Hind*III-digested pSC146 (204 base transcript) with σ^K RNA polymerase alone (lane 5) or with SpoIIID added (lane 6).

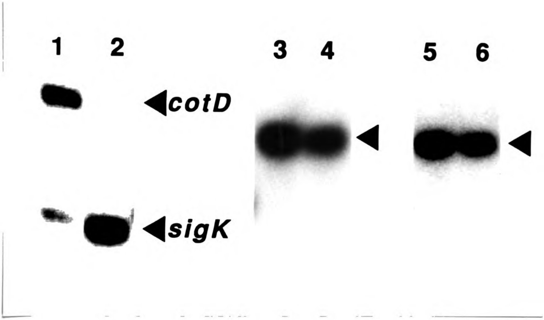
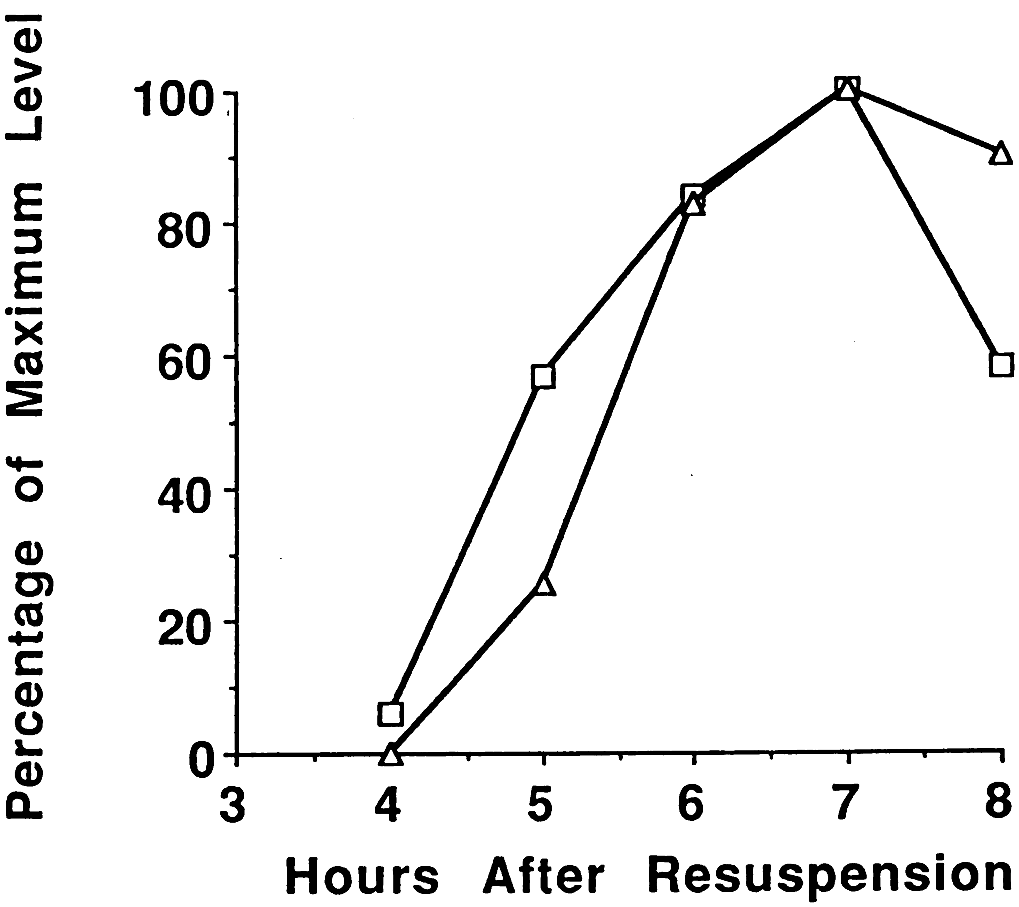

1
2
3
4
5
6
cotD
sigK

Figure 6. Levels of *gerE* and *cotD* mRNA in sporulating *B. subtilis*. *spo*$^+$ cells were sporulated by the resuspension method. RNA was prepared from cells collected between T_4 and T_8 and subjected to Northern blot analysis. The signals obtained for *cotD* (Δ) and *gerE* (❐) mRNA were quantitated using a Visage Digital Imager and plotted as the percentage of the maximum level achieved during sporulation.


Percentage of Maximum Level
100
80
60
40
20
0
3
4
5
6
7
8
Hours After Resuspension

upstream of the *cotD* transcriptional start site to codon 58 of the *cotD* ORF. A similar result to that obtained with the 430 bp *cotD* probe was also obtained utilizing a nick-translated 169 bp *Pst*I fragment from pLRK100 (Kroos *et al.,* 1989) which contains codons 4 through 59 of the *cotD* ORF. mRNAs of approximately 400 bases were detected with all three probes (data not shown). This observation is in good agreement with the size of *gerE* mRNA that was reported previously (Cutting & Mandelstam, 1986). The size of *cotD* mRNA suggests that it is monocistronic. *gerE* mRNA is first detected at T_4 and its level increased significantly between T_4 and T_5 , whereas *cotD* mRNA is first detected at T_5 and its level increased significantly between T_5 and T_6 (Figure 6). This difference in timing was observed in two additional experiments (data not shown). The delay in *cotD* expression may be caused by SpoIIID-mediated repression.

Discussion

While attempting to determine whether SpoIIID represses *cotD* expression during sporulation, we found that overproduction of SpoIIID reduced the level of σ^K. This was surprising since SpoIIID plays a positive role in several aspects of σ^K production, including the chromosomal rearrangement that generates the intact *sigK* gene (Stragier *et al.*, 1989; Kunkel *et al.*, 1990), the transcription of *sigK* initially by σ^E RNA polymerase (Chapter II) and later by σ^K RNA polymerase (Kunkel *et al.*, 1988; Kroos *et al.*, 1989), and processing of pro-σ^K to σ^K (S. Lu & L. Kroos, unpublished data). Overproduction of SpoIIID does not appear to interfere with the initial transcription of *sigK* by σ^E RNA polymerase, the chromosomal rearrangement that generates *sigK*, or translation of *sigK* mRNA to produce pro-σ^K. Transcription of *sigK* by σ^E RNA polymerase actually appears to be enhanced in cells overproducing SpoIIID since *sigK*-directed ß-galactosidase activity is slightly elevated at T_3 and T_4 (i.e., the time interval when SpoIIID (Halberg & Kroos, 1992) and σ^E (Trempy *et al.*, 1985b) are both present) as compared to wild-type cells (Figure 2A). This suggests that SpoIIID is limiting for *sigK* transcription by σ^E RNA polymerase in wild-type cells. The chromosomal rearrangement and translation of *sigK* mRNA appear to be unaffected by SpoIIID overproduction because the amount of pro-σ^K present between T_3 and T_8 is comparable in wild-type cells and cells overproducing SpoIIID (Figure 2B).

Overproduction of SpoIIID does appear to interfere with the processing of pro-σ^K to σ^K and/or decrease the stability of σ^K in sporulating cells. The amount of σ^K present at T_4 is lower in cells overproducing SpoIIID than in wild-type cells (Figure 2B). Deficient processing of pro-σ^K and/or decreased stability of σ^K during early times in sporulation would be expected to reduce the level of *sigK* promoter activity during late times in sporulation (i.e., T_5 to T_8), as was observed (Figure 2A), because *sigK* is transcribed by σ^K RNA polymerase (Kunkel *et al.*, 1988; Kroos *et al.*, 1989). Reduced *sigK* promoter activity during late times in sporulation would not necessarily result in lower pro-σ^K levels in cells overproducing SpoIIID (e.g., a defect in processing would tend to elevate the level of pro-σ^K), but the total amount of pro-σ^K and σ^K present should be less than in wild-type cells, as was observed (Figure 2B). SpoIIID positively or negatively regulates the expression of many genes, including *bofA* (Ireton & Grossman, 1992a; Ricca *et al.*, 1992), *spoIIIA* (Illing & Errington, 1991b), *spoIIIG* (Karmazyn-Campelli *et al.*, 1989), *spoIVB* (Cutting *et al.*, 1991a), and *spoIVF* (Cutting *et al.*, 1991b), whose products influence pro-σ^K processing (Cutting *et al.*, 1990; Lu *et al.*, 1990). The requirement for *spoIIIA*, *spoIIIG* and *spoIVB*, but not for *spoIVF*, in processing can be bypassed by *bof* mutations. However, a *bof* mutation did not overcome the negative affect of SpoIIID overproduction on σ^K accumulation (data not shown). Overproducing SpoIIID may reduce the level of σ^K by altering the expression of *spoIVF*, *bofA*, and/or other genes involved in pro-σ^K processing and/or σ^K stability.

Maintaining the proper levels of SpoIIID and σ^K during sporulation is critical because the overproduction of SpoIIID and/or the resulting reduction in the level of σ^K markedly reduced the formation of heat-resistant spores (Table 1). A lowered level of σ^K RNA polymerase is expected to reduce expression of genes involved in spore cortex (Cutting *et al.*, 1991a) and coat formation (Zheng & Losick, 1990; Zheng *et al.*, 1992). Thus, altered expression of many genes in the mother cell presumably contributes to the reduction in heat-resistant spore formation by cells overproducing SpoIIID.

Normally, SpoIIID production, like σ^K production, is subject to multiple levels of regulation. First, the synthesis of *spoIIID* mRNA appears to be positively autoregulated since *spoIIID*-directed ß-galactosidase activity is reduced 3- to 6-fold in a *spoIIID* mutant background (Kunkel *et al.*, 1989; Stevens & Errington, 1990). Second, the synthesis and/or stability of *spoIIID* mRNA is negatively regulated by the production of σ^K (Halberg & Kroos, 1992). Third, *spoIIID* mRNA is polycistronic, and translation through the first ORF appears to be required for translation of the *spoIIID* ORF since a mutation in the ribosomal binding site of the first ORF reduces the expression of SpoIIID-dependent genes (A. Bosma & R. Losick, unpublished data). Fourth, SpoIIID appears to be rapidly degraded in the mother cell during late times in sporulation since a decrease in the level of *spoIIID* mRNA is paralleled by a decrease in the level of SpoIIID (Halberg & Kroos, 1992). The degradation of SpoIIID may involve the conversion of SpoIIID to a less stable intermediate (Chapter IV). This conversion appears to be developmentally regulated and involves the removal of amino acids from the C-terminal portion of SpoIIID (Chapter IV). All of these levels of regulation provide potential mechanisms for connecting the level of SpoIIID, and hence the expression of many mother-cell genes, to genetic regulatory, physiological, and morphological cues during the sporulation process.

We used cells engineered to make σ^K during growth to demonstrate that SpoIIID can stimulate *sigK* and inhibit *cotD* expression, but does not affect *cotA* or *gerE* expression *in vivo* (Figures 3 and 4). The effects of SpoIIID on the transcription of all four genes tested in this *in vivo* system are, at least qualitatively, consistent with the results of *in vitro* transcription assays (Figure 5). This system provides a convenient method for examining the effects *in vivo* of SpoIIID on expression of σ^K-dependent genes. The slopes of the curves are noteworthy because they may reflect accumulation of GerE in cells. GerE stimulates *cotD* transcription (Zheng & Losick, 1990; Zheng *et al.*, 1992) and *cotD-lacZ* expression increased with time after induction of σ^K production (Figure 4A), whereas GerE inhibits *cotA* transcription (Sandman *et al.*, 1988; Cutting *et al.*, 1989; Zheng *et al.*, 1992) and *cotA-lacZ* expression decreased with time after induction (Figure 4B). Expression of *gerE-lacZ* (Figure 4C) and *sigK-lacZ* (Figure 3B) changed little with time, consistent with the absence of a *gerE* effect on the expression of these fusions (Kunkel *et al.*, 1988; Cutting *et al.*, 1989). The *sigK-lacZ* fusion lacks a site for GerE binding that mediates the strong repression of *sigK* transcription by GerE observed *in vitro* (H. Ichikawa and L. Kroos, unpublished data; Zheng *et al.*, 1992).

While our results demonstrate that SpoIIID can repress *cotD* transcription in cells engineered to produce σ^K during growth, the question of whether SpoIIID represses *cotD* transcription during sporulation, thus altering its time or level of expression, remains unanswered. The finding that SpoIIID can repress *cotD* transcription by σ^K RNA polymerase *in vitro* originally led to the proposal that SpoIIID represses *cotD* transcription for a period during sporulation (Kroos *et al.*, 1989). We subsequently showed that induction of a *cotD-lacZ* fusion coincides with a decrease in the level of SpoIIID during sporulation in wild-type cells (Halberg & Kroos, 1992). Furthermore, *cotD-lacZ* induction coincides with a decrease in the level of SpoIIID but lags behind an increase in the level of σ^K in cells engineered to produce σ^K earlier than normal; this was shown not to be an effect of GerE (which stimulates *cotD* transcription), suggesting that *cotD-lacZ* induction was delayed until the level of SpoIIID decreased (Halberg & Kroos, 1992). In contrast, the induction of *cotA*- and *gerE-lacZ* fusions coincides with the σ^K increase in these cells and precedes the SpoIIID decrease (R. Halberg and L. Kroos, unpublished data), suggesting that transcription of *cotA* and *gerE* is not subject to repression by SpoIIID. Consistent with this idea is the finding that SpoIIID's ability to repress *cotA* and *gerE* transcription by σ^K RNA polymerase *in vitro* is weak relative to its ability to repress *cotD* transcription (Figure 5). Moreover, the major increase in *cotA*- and *gerE-lacZ* expression during sporulation occurs one hour earlier than for *cotD-lacZ* ((Oke & Losick, 1993); Figure 1) and the appearance of *gerE* mRNA also precedes the appearance of *cotD* mRNA by about one hour (Figure 6). All these results are consistent with the model that SpoIIID represses *cotD* transcription, but not *cotA* or *gerE* transcription, for about one hour during sporulation.

If SpoIIID does repress *cotD* transcription, but not *cotA* or *gerE* transcription, during sporulation, one might have expected an elevated level of SpoIIID to exert a more pronounced negative effect on *cotD-lacZ* expression than on *cotA* or *gerE-lacZ* expression; however, this was not observed (Figure 1). Apparently, even the elevated level of SpoIIID is insufficient to markedly reduce *cotD* transcription late in sporulation (i.e., at T_6 to T_8). The timing of *cotD-lacZ* expression was unaffected by SpoIIID overproduction, remaining about one hour later than *cotA*- and *gerE-lacZ* expression (Figure 1). Thus, it remains possible that SpoIIID prevents premature transcription of *cotD* for a short period during sporulation while the levels of σ^K RNA polymerase and GerE are low (Halberg & Kroos, 1992). An alternative explanation for the later expression of *cotD* is that a higher threshold level of σ^K may be required for *cotD* transcription than for transcription of *cotA* and *gerE* (Oke &

Losick, 1993). *cotD* is expressed about 1 to 2 hours later than *gerE* when σ^K production is induced with IPTG during growth in cells containing P*spac*-P*sigK*-*sigK*Δ*19* (Oke & Losick, 1993). Since SpoIIID is absent in this case, it cannot account for the difference in timing. We have mapped a site for SpoIIID binding in the *cotD* promoter region (Chapter II). Perhaps mutations that eliminate SpoIIID binding in the *cotD* promoter region will enable us to determine whether SpoIIID affects *cotD* transcription during sporulation.

SpoIIID is just one component of a molecular switch proposed to govern the change in the mother-cell pattern of gene expression during the transition from morphological stage IV (cortex formation) to stage V (coat formation) (Halberg & Kroos, 1992; Zheng *et al.*, 1992). Two other components are σ^K RNA polymerase and GerE. Processing of pro-σ^K to σ^K appears to activate the switch, resulting in a falling level of SpoIIID due (at least in part) to a falling level of *spoIIID* mRNA (Halberg & Kroos, 1992) and a rising level of GerE due to transcription of *gerE* by σ^K RNA polymerase (Zheng *et al.*, 1992). SpoIIID and GerE exert opposite effects on transcription of certain genes *in vitro*. For example, transcription of the earlier-expressed *sigK* gene is stimulated by SpoIIID (Kunkel *et al.*, 1988; Kroos *et al.*, 1989, Figure 5) and inhibited by GerE (Zheng *et al.*, 1992), whereas transcription of the later-expressed *cotD* gene is inhibited by SpoIIID (Kroos *et al.*, 1989, Figure 5) and stimulated by GerE (Zheng *et al.*, 1992). Hence, both a falling level of SpoIIID and a rising level of GerE would change the pattern from *sigK* to *cotD* transcription by σ^K RNA polymerase.

Why the need for this elaborate switch involving dual control by SpoIIID and GerE? One clue comes from the observation that other *cot* genes (encoding spore coat proteins) may also be subject to dual control by SpoIIID and GerE. Transcription of *cotC* and *cotX* by σ^K RNA polymerase, like that of *cotD*, is inhibited by SpoIIID and stimulated by GerE *in vitro* (H. Ichikawa & L. Kroos, unpublished data). We speculate that differing arrangements and affinities of binding sites for SpoIIID and GerE in *cot* promoters may influence the time and level of production of different spore coat proteins to facilitate assembly of the multilayered spore coat.

In summary, we have shown that overproduction of SpoIIID reduces the level of σ^K during sporulation and reduces *cotD* expression during vegetative growth. Overproduction of SpoIIID probably reduced the level of σ^K during sporulation by altering the expression of genes encoding proteins involved in pro-σ^K processing and/or σ^K stability. The reduced level of σ^K lowered the expression of all σ^K-dependent genes tested. Maintaining the proper levels of SpoIIID and σ^K is crucial for normal sporulation because the overproduction of SpoIIID and/or the resulting reduction in the level of σ^K reduced the production of heat-resistant spores. This is probably why the production of both SpoIIID and σ^K is normally subject to multiple levels of regulation. The problem of SpoIIID overproduction reducing the level of σ^K during sporulation was circumvented by engineering cells to produce σ^K during vegetative growth. Expressing SpoIIID from P*spac-spoIIID* in this strain increased *sigK* promoter activity and reduced *cotD* promoter activity, but did not affect *cotA* or *gerE* promoter activity, in agreement with the effects of SpoIIID on transcription of these promoters *in vitro*. Clearly, *cotD* is expressed about one hour later than *gerE* and *cotA* during sporulation, but measuring the relative contributions of a falling level of SpoIIID and a rising level of GerE to the timing of *cotD* expression, and understanding the biological significance of this finely tuned regulation, will require further investigation.

CHAPTER VI

Conclusions

"The end justifies the means only when the means used are such as actually bring about the desired and desirable end."

John Dewey

The goal of my research was to characterize the role of SpoIIID during *Bacillus subtilis* sporulation. Experiments were performed to determine the transcriptional properties and fate of SpoIIID.

Genetic studies suggested that SpoIIID affects the transcription by σ^E RNA polymerase (reviewed by Errington, 1993). To determine whether this was a direct effect, *in vitro* transcription assays were performed. SpoIIID stimulated *spoIVCA* and *sigK* transcription, but represses *bofA* transcription, by σ^E RNA polymerase. DNase I footprinting revealed that SpoIIID binds to specific sequences in the promoter regions and open reading frames (ORFs) of these genes.

Other studies demonstrated that SpoIIID stimulates *sigK* transcription, but represses *cotD* transcription, by σ^K RNA polymerase *in vitro* (Kroos *et al.*, 1989). Consistent with this observation, the presence of SpoIIID reduced the level of ß-galactosidase generated from a *cotD-lacZ* fusion in cells engineered to produce σ^K during vegetative growth, indicating that SpoIIID can repress *cotD* expression *in vivo*. SpoIIID binds to the promoter region and ORF of both *sigK* and *cotD*. However, SpoIIID binding in the promoter region is sufficient to mediate the transcriptional effects observed *in vitro*.

Comparison of the sequences in the SpoIIID binding sites of σ^E and σ^K-dependent genes revealed a putative consensus, WWRRACAR-Y. This consensus sequence is centered at -28 and -27 in *spoIVCA* and *sigK*, respectively, but at -33 in *cotD*, suggesting that SpoIIID binds on opposite sides of the DNA helix (relative to RNA polymerase) in promoters where it exerts opposite effects on transcription. The spatial orientation of SpoIIID relative to RNA polymerase would be expected to affect the interaction between these proteins. In the case of *spoIVCA* and *sigK*, SpoIIID may interact with the α or σ subunits of RNA polymerase, as has been observed for other transcriptional activators. In the case of *cotD*, SpoIIID may block the interaction between the -35 region of the *cotD* promoter and σ^K RNA polymerase. Additional genetic and biochemical experiments may provide further insight into how SpoIIID acts as a transcriptional activator and repressor.

Based on the observation that SpoIIID activates and represses transcription by σ^K RNA polymerase, it was proposed that the inactivation and/or sequestering of SpoIIID establishes a switch in the mother-cell pattern of gene expression. Western blot analysis demonstrated that the level of SpoIIID does decrease at the appropriate time during sporulation to produce such a switch. This decrease is dependent upon the production of σ^K. Northern blot analysis revealed that the production of σ^K reduced the synthesis and/or stability of *spoIIID* mRNA. This could be a direct or an indirect effect. σ^K may compete with σ^E for binding to core RNA polymerase and thereby reduce the level of σ^E RNA polymerase. Consistent with this idea, σ^E appears to be a labile protein. Alternatively, σ^K RNA polymerase may transcribe a gene whose product represses spoIIID transcription. These possibilities can be distinguished by mutating *sigK* such that the σ^K produced can bind to core RNA polymerase but is transcriptionally inactive, or it can bind to DNA but is unable to bind to core RNA polymerase.

The parallel between the decrease in the levels of *spoIIID* mRNA and SpoIIID protein, suggests that a mechanism exists for degrading SpoIIID in sporulating cells. Consistent with this idea, SpoIIID appears to be converted to a less stable 9 kDa form by removing 7 amino acids from its C-terminus, based on N-terminal sequencing and mass spectral analysis. However, additional mass spectral experiments are required to determine the C-terminus of the 9 kDa protein conclusively and a pulse-chase experiment is required to demonstrate a precursor-product relationship between SpoIIID and the 9 kDa protein. The conversion of SpoIIID to the 9 kDa protein is developmentally regulated. Interestingly, mutations in genes required for the formation of the spore cortex inhibit the production of the 9 kDa protein, suggesting the conversion may be coupled to spore morphogenesis. For example, the conversion is blocked by a mutation in *spoIVA*, which encodes a novel 50 kDa protein that is required for cortex and coat formation. It remains to be determined if the SpoIVA protein is responsible for the conversion. A mobility shift system is ready to monitor the levels of SpoIIID and the 9 kDa protein during attempts to purify the convertase. Specific proteolysis plays a role in controlling gene expression in other systems.

The proper level of SpoIIID is crucial for normal sporulation. Cells engineered to produce an elevated level of SpoIIID throughout sporulation produced fewer heat-resistant spores. This effect appeared to result from a reduction in the level of σ^K and σ^K-dependent gene expression. Several experiments were performed to determine how the overproduction of SpoIIID reduced the level of σ^K. Unfortunately, the results were inconclusive.

Sigma factors, anti-sigma factors, and DNA-binding proteins are present in other organisms. Continued characterization of the role of SpoIIID during *B. subtilis* sporulation may reveal basic mechanisms by which DNA-binding proteins regulate gene expression during development.

APPENDICES

APPENDIX A

Processing of the Mother-Cell σ Factor, σ^K, May Depend On Events Occurring in the Forespore During *Bacillus subtilis* Development

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Processing of the mother-cell σ factor, σ^K, may depend on events occurring in the forespore during *Bacillus subtilis* development

(gene expression/proteolysis/RNA polymerase/sporulation/transcription)

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ABSTRACT **During sporulation of the Gram-positive bacterium *Bacillus subtilis*, transcription of genes encoding spore coat proteins in the mother-cell compartment of the sporangium is controlled by RNA polymerase containing the σ subunit called σ^K. Based on comparison of the N-terminal amino acid sequence of σ^K with the nucleotide sequence of the gene encoding σ^K (*sigK*), the primary product of *sigK* was inferred to be a pre-protein (pro-σ^K) with 20 extra amino acids at the N terminus. Using antibodies generated against pro-σ^K, we have detected pro-σ^K beginning at the third hour of sporulation and σ^K beginning about 1 hr later. Even when pro-σ^K is expressed artificially during growth and throughout sporulation, σ^K appears at the normal time and expression of a σ^K-controlled gene occurs normally. These results suggest that pro-σ^K is an inactive precursor that is proteolytically processed to active σ^K in a developmentally regulated fashion. Mutations that block forespore gene expression block accumulation of σ^K but not accumulation of pro-σ^K, suggesting that pro-σ^K processing is a regulatory device that couples the programs of gene expression in the two compartments of the sporangium. We propose that this regulatory device ensures completion of forespore morphogenesis prior to the synthesis in the mother-cell of spore coat proteins that will encase the forespore.**

Upon starvation the Gram-positive bacterium *Bacillus subtilis* undergoes a series of morphological changes that result in endospore formation (1). The first easily observed morphological change is asymmetric septum formation, which divides the cell into two compartments, the mother-cell and the forespore, each receiving a copy of the genome. A complex regulatory circuit ensures the correct temporal and spatial pattern of gene expression during sporulation. Critical to this regulatory circuit are the synthesis and activation of σ subunits of RNA polymerase that direct the enzyme to transcribe different gene sets (2). Two σ factors are compartment-specific. σ^G, the product of the *spoIIIG* gene, is produced predominantly, if not exclusively, in the forespore and controls the expression of forespore-specific genes (3–5). The counterpart to σ^G in the mother-cell is σ^K (6), which controls the expression of mother-cell-specific genes such as *cotA* (7), *cotD* (8), and *gerE* (9) (referred to as the *cotA* regulon). The *cotA* and *cotD* genes encode spore coat proteins that assemble on the forespore surface (10), and *gerE* encodes a regulator of spore coat synthesis (11).

σ^K is encoded in a composite gene (*sigK*) generated by a mother-cell-specific chromosomal rearrangement that joins two loci, *spoIVCB* (encoding the N-terminal portion) and *spoIIIC* (encoding the C-terminal portion) (12, 13). Transcription of the *sigK* promoter is also confined to the mother-cell (14) and compartmentalization of both the DNA rearrangement and *sigK* transcription appear to result from mother-cell-specific expression of *spoIIID* (15). A third possible regulatory mechanism for σ^K was inferred from a comparison of the N-terminal amino acid sequence of σ^K and the nucleotide sequence of *sigK* (6, 12) and by analogy to σ^E, a sporulation-specific *B. subtilis* σ factor that is activated by proteolytic processing (16–18). The primary product of *sigK* was predicted to be a pro-protein (pro-σ^K) bearing 20 extra amino acids at the N terminus. Here we present evidence that σ^K is first made as an inactive precursor and is processed to the active σ factor in a developmentally regulated fashion. Furthermore, mutations in forespore regulatory genes (e.g., *spoIIIG*, encoding the forespore σ factor, σ^G) appear to block processing of pro-σ^K to σ^K, suggesting that the previously noted dependence of mother-cell-specific gene expression on forespore events (7–9, 14) is mediated at the level of proteolytic activation of the mother-cell σ factor.

MATERIALS AND METHODS

Bacterial Strains. *Escherichia coli* strain AG115 [*araD139*, Δ(*ara*, *leu*)*7697*, Δ*lacX74*, *galU*$^-$, *galK*$^-$, *hsr*$^-$, *hsm*$^+$, *strA*, (F′, *proAB*, *lacI*^{q}Z::Tn*5*)] was obtained from A. Grossman. *B. subtilis* strains were obtained from R. Losick. *B. subtilis* cells were made competent (19) and transformants were selected on Luria–Bertani (LB) agar (20) with kanamycin sulfate at 5 μg/ml. Use of the specialized transducing phage SPβ::*cotD–lacZ* (obtained from L. Zheng and R. Losick) has been described (8).

Construction of Plasmids. All plasmids were derived from pSK5, which contains *sigK* (13), and from pDG148 (18), which permits isopropyl β-D-thiogalactopyranoside (IPTG)-inducible expression of an inserted gene from the P_{spac} promoter (21) in *E. coli* or *B. subtilis*. To fuse *sigK* expression to the P_{spac} promoter, a 1.4-kilobase-pair (kbp) *Ssp* I–*Hin*dIII fragment from pSK5 (including 141 bp upstream and 556 bp downstream of the *sigK* open reading frame) was ligated to *Hin*dIII-digested pDG148, the unligated end of the vector was made blunt using the fill-in reaction of Klenow enzyme, and ligation was continued (20). Ampicillin-resistant *E. coli* transformants were obtained (20) and the structure of pSL1, a plasmid containing the insert in the proper orientation to fuse *sigK* transcription to P_{spac}, was verified by restriction mapping. pSL2 and pSL4 were derived from pSL1 and pDG148, respectively, by deletion of the *Eco*RI fragment containing the origin of replication and the kanamycin-resistance gene that function in *B. subtilis*.

Production of Pro-σ^K and Preparation of Antibodies. To produce pro-σ^K in *E. coli*, strain ESL2 (strain AG115 containing pSL2) was induced with 1 mM IPTG during the late logarithmic phase of growth at 37°C in LB medium (22). One hour after the addition of IPTG, cells were harvested by

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Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; T_n, hour *n* of sporulation.

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centrifugation (6 min, 7500 × *g*). The cell pellet was resuspended in 0.05 volume of sample buffer (0.125 M Tris·HCl, pH 6.8/2% SDS/5% 2-mercaptoethanol/10% glycerol/0.1% bromophenol blue) and the sample was boiled for 5 min to produce a whole-cell extract. From 0.5 ml of extract, ~100 μg of pro-σ^K was purified by preparative SDS/PAGE (10–15% polyacrylamide gradient) and electroelution. Pro-σ^K (65 μg) was precipitated with acetone, dissolved in phosphate-buffered saline (23), emulsified with Freund's complete adjuvant (BRL), and injected into or near the popliteal gland of a New Zealand White rabbit. Three weeks later a booster injection [30 μg of pro-σ^K emulsified with Freund's incomplete adjuvant (BRL)] was given at the same site. The rabbit was bled 1 week after the boost and serum was prepared (23).

Sporulation and Western Blot Analysis. Sporulation of *B. subtilis* was initiated by nutrient exhaustion in Difco sporulation (DS) medium (24) as described (7). Cells were harvested by centrifugation (5 min, 16,000 × *g*) and whole-cell extracts were prepared (22). Extract protein was quantitated by the Bradford method (25). After addition of 0.5 volume of 3× sample buffer, proteins were separated by SDS/12.5% PAGE and electroblotted to a poly(vinylidene difluoride) membrane (26). The membrane was incubated in TBS (20 mM Tris·HCl, pH 7.5/0.5 M NaCl) with 2% nonfat dry milk for 4 hr at room temperature with shaking to block nonspecific antibody binding and then incubated overnight at room temperature with shaking in polyclonal antiserum diluted 1:2000 into TBS/2% nonfat dry milk/0.05% Tween 20. Immunodetection using a goat anti-rabbit alkaline phosphatase conjugate was performed according to the manufacturer's instructions (Bio-Rad).

RESULTS

Antibodies to Pro-σ^K Detect Pro-σ^K and σ^K in Sporulating *B. subtilis*. To purify pro-σ^K for the generation of a polyclonal antiserum, the protein was expressed in *E. coli*. Transcription of *sigK* (encoding pro-σ^K) was fused to an IPTG-inducible promoter in plasmid pSL2. Whole-cell extracts of IPTG-induced and uninduced *E. coli* containing pSL2 or a control plasmid (pSL4, which does not contain *sigK*) were analyzed by gel electrophoresis (Fig. 1). A protein of the expected mobility for pro-σ^K [~29 kDa, since pro-σ^K is predicted to contain 20 amino acids at its N terminus that are absent from σ^K, which migrates at 27 kDa (6)] increased upon IPTG induction of cells containing pSL2, but not upon IPTG induction of cells containing the control plasmid. This protein was assumed to be pro-σ^K, the predicted primary translation product of *sigK*, since pSL2 contains no other 29-kDa-protein-encoding open reading frames downstream of the IPTG-inducible promoter.

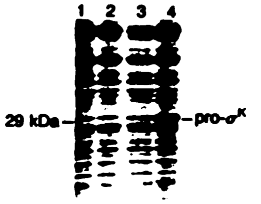

FIG. 1. Production of pro-σ^K in *E. coli*. Proteins in whole-cell extracts (10 μl) of IPTG-induced (lanes 2 and 4) and uninduced (lanes 1 and 3) *E. coli* were separated by SDS/PAGE (10–15% polyacrylamide gradient) and visualized by Coomassie blue staining. Strains ESL4 (lanes 1 and 2) and ESL2 (lanes 3 and 4) were constructed by transformation of strain AG115 with the control plasmid (pSL4) and the P_{spac}–*sigK* fusion plasmid (pSL2), respectively. Only the 35- to 25-kDa region of the gel is shown; the positions of a 29-kDa marker protein (carbonic anhydrase) and the protein assumed to be pro-σ^K are indicated.

Antibodies to gel-purified pro-σ^K were generated in a rabbit and used in Western blot analyses. The antibodies detected pro-σ^K and one larger protein in a whole-cell extract of IPTG-induced *E. coli* containing pSL2 (Fig. 2*A*, lane 2), while only the larger protein was detected for cells containing the control plasmid without *sigK* (lane 1). The antibodies, referred to hereafter as "anti-pro-σ^K antibodies," easily detected 15 ng of pro-σ^K gel-purified from *E. coli* (Fig. 2*B*, lane 1). The anti-pro-σ^K antibodies also recognized σ^K (6) gel-purified from *B. subtilis* (Fig. 2*B*, lane 2) and these antibodies detected either pro-σ^K or σ^K with similar sensitivity. The antibodies detected proteins that comigrated with pro-σ^K and σ^K in a whole-cell extract of sporulating *B. subtilis* (Fig. 2*B*, lane 3), while these proteins were not detected in extracts of growing *B. subtilis* (lane 4) or in extracts of *sigK* mutants (i.e., *spoIVCB* or *spoIIIC* mutants; see below). Thus, Western blot analysis using the anti-pro-σ^K antibodies provides a sensitive assay for the level of pro-σ^K and σ^K in *B. subtilis*.

Levels of Pro-σ^K and σ^K Are Developmentally Regulated. To examine the levels of pro-σ^K and σ^K in *B. subtilis* at various times during sporulation, cells were harvested at hourly intervals during growth and sporulation in DS medium. Under these conditions, the end of exponential growth defines the initiation of sporulation (T_0), prespores that appear gray in the phase-contrast microscope begin to appear 4 hr later (T_4), and phase-bright free spores (released by mother-cell lysis at the end of sporulation) begin to appear at T_8. Whole-cell extracts were subjected to Western blot analysis using the anti-pro-σ^K antibodies and the result for the Spo$^+$ strain PY79 (27) is shown in Fig. 3. A similar result was obtained for the Spo$^+$ strain SG38 (28) (data not shown). Pro-σ^K was first observed at 3 hr into the sporulation process (T_3), reached a maximum at T_5, and then decreased to a barely detectable level by T_8. σ^K was first observed at T_4 (1 hr later than pro-σ^K), increased to a maximum at T_6, and decreased thereafter. These results demonstrate that the levels of pro-σ^K and σ^K are regulated during sporulation. Since the appearance of pro-σ^K precedes the appearance of σ^K and since the N terminus of σ^K corresponds to codon 21 of *sigK* (6, 12), σ^K may be derived from pro-σ^K by proteolytic processing.

Mutations in Many Sporulation Genes Block Accumulation of σ^K. Mutations at many different loci in the *B. subtilis*

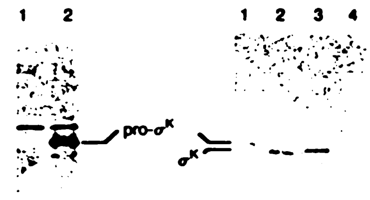

FIG. 2. Characterization of the anti-pro-σ^K antiserum by Western blot analyses. (*A*) Whole-cell extracts (1 μl of a 1:100 dilution) from IPTG-induced *E. coli* strains ESL4 (lane 1) and ESL2 (lane 2), containing the control plasmid (pSL4) and the P_{spac}–*sigK* fusion plasmid (pSL2), respectively, were prepared as described for the production of pro-σ^K (see *Materials and Methods*). (*B*) Pro-σ^K (15 ng) from *E. coli* (lane 1) and σ^K (15 ng) from sporulating *B. subtilis* (lane 2) were gel-purified. Whole-cell extracts (10 μg of protein) were from *B. subtilis* harvested during growth (lane 4) and at 6 hr into sporulation (lane 3) in DS medium.

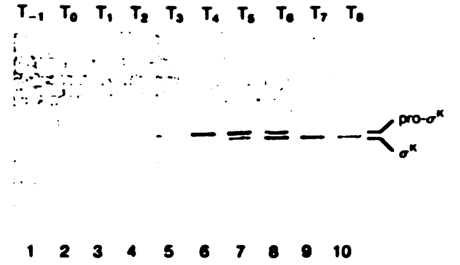

FIG. 3. Pro-σ^K and σ^K in sporulating *B. subtilis*. Wild-type strain PY79 (27) was harvested at hourly intervals during growth and sporulation in DS medium. Whole-cell extracts (10 μg of protein) were subjected to Western blot analysis using the anti-pro-σ^K antibodies. Lanes 1–10, samples harvested at hourly intervals beginning 1 hr before the end of exponential growth (T_{-1}) and ending 8 hr into sporulation (T_8). Pro-σ^K gel-purified from *E. coli* served as a marker on the blot and the inferred position of σ^K is indicated.

genome block or reduce expression of genes in the σ^K-controlled *cotA* regulon (7–9). To further investigate these effects, 16 mutants with altered *cotA* regulon expression were examined for pro-σ^K and σ^K by using the anti-pro-σ^K antibodies in Western blot analyses. Samples collected at hourly intervals from T_0 through T_7 in DS medium were tested for each mutant, but only the result at T_6, the time when both pro-σ^K and σ^K are abundant in the Spo$^+$ strains (see above), is shown in Fig. 4 for each mutant. Five mutants accumulated neither pro-σ^K nor σ^K (lanes 3, 5, 6, 12, and 13). These mutants have either a mutation in the *sigK* gene [*spoIVCB* and *spoIIIC* (12)] or a mutation in a gene whose product is essential for the chromosomal rearrangement that generates *sigK* [*spoIIGB*, *spoIIID*, and *spoIVCA* (12, 13)]. Nine mutants accumulated pro-σ^K but not σ^K (lanes 1, 2, 4, 7, 8, 11, 14, 15, and 16). Interestingly, this group includes 4 strains with mutations in genes required for forespore-specific gene expression [*spoIIIA*, *spoIIIE*, and *spoIIIG* (32, 33)] and/or in genes expressed predominantly, if not exclusively, in the forespore [*spoIIIG* (4, 5) and *spoIVB* (S. Cutting and R. Losick, personal communication)], suggesting that accumulation of σ^K in the mother-cell compartment of the sporangium depends on events occurring in the forespore compartment. In addition, this group includes 2 mutants (*spoIIB* and *spoIID*) blocked early in sporulation at the stage of asymmetric septum formation and 3 strains with mutations in the *spoIVF* locus, which is not required for expression of a forespore-specific gene (32). Finally, 2 strains with mutations in *spoIVA* accumulated a normal amount of pro-σ^K but accumulated much less σ^K (lanes 9 and 10) than the wild-type strain. The *spoIVA* mutants express the *cotA* regulon at a reduced level, whereas all the other mutants examined in this study fail to express the *cotA* regulon (7–9). Thus, for all the mutants examined, the impaired *cotA* regulon expression observed previously (7–9) may be due to impaired accumulation of σ^K. If σ^K is derived from pro-σ^K by proteolytic processing as suggested above, at least eight loci (*spoIIB*, *spoIID*, *spoIIIA*, *spoIIIE*, *spoIIIG*, *spoIVA*, *spoIVB*, and *spoIVF*) may be directly or indirectly involved in processing pro-σ^K and/or stabilizing σ^K.

Processing of Pro-σ^K to σ^K Is Required to Produce an Active σ Factor and Is Developmentally Regulated. *In vitro* and *in vivo* approaches were used to address whether pro-σ^K can direct transcription of σ^K-controlled promoters. For the *in vitro* approach, pro-σ^K gel-purified from *E. coli* was tested for its ability to direct transcription of the *sigK* [previously called *spoIVCB* (6, 14)] and *cotD* (8) promoters upon addition to *B. subtilis* core RNA polymerase (6). As a positive control, σ^K (60 ng) partially purified from sporulating *B. subtilis* was eluted from a gel, renatured (34), and added to core RNA polymerase (60 ng). The reconstituted enzyme produced a run-off transcript from the *sigK* promoter in the presence of the SpoIIID protein (120 ng) and from the *cotD* promoter in the absence of SpoIIID (data not shown), as shown previously (6). Under these conditions, pro-σ^K (300 ng) failed to direct transcription of the *sigK* promoter in the presence of SpoIIID (120 ng) and also failed to direct transcription of the *cotD* promoter in the absence of SpoIIID (data not shown). These results suggest that pro-σ^K is inactive as a σ factor.

To determine whether pro-σ^K could direct transcription of a σ^K-controlled gene *in vivo*, we used a multicopy plasmid bearing *sigK* fused to an IPTG-inducible promoter to express pro-σ^K in *B. subtilis* during growth and sporulation and a *cotD–lacZ* fusion (8) to monitor the transcriptional activity of a σ^K-controlled promoter. A *sigK* mutation prevented production of pro-σ^K or σ^K from the chromosome in this experiment. Production of pro-σ^K from the plasmid was induced with IPTG ∼2 hr before the end of exponential growth, and samples collected at hourly intervals were tested for β-galactosidase production from the *cotD–lacZ* fusion and were also subjected to Western blot analysis using the anti-pro-σ^K antibodies (Fig. 5). Even though a large amount of pro-σ^K was present 1 hr prior to the end of exponential growth (T_{-1}) and throughout the early stages of sporulation (Western blot, *Inset*), *cotD*-directed β-galactosidase activity remained low

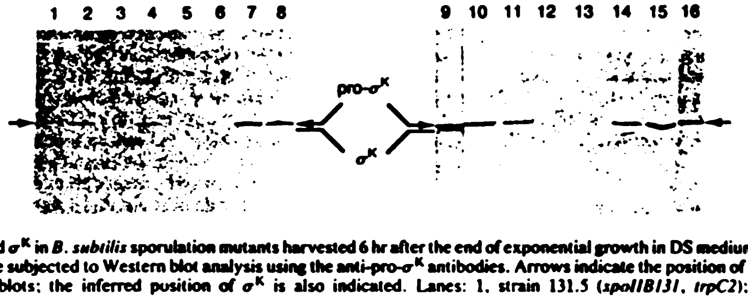

FIG. 4. Pro-σ^K and σ^K in *B. subtilis* sporulation mutants harvested 6 hr after the end of exponential growth in DS medium. Whole-cell extracts (10 μg of protein) were subjected to Western blot analysis using the anti-pro-σ^K antibodies. Arrows indicate the position of pro-σ^K, which served as a marker on the blots; the inferred position of σ^K is also indicated. Lanes: 1, strain 131.5 (*spoIIB131*, *trpC2*); 2, KS298 (*spoIID*::Tn*917*ΩHU*298*); 3, KS440 (*spoIIG41*); 4, KS13 (*spoIIIA*::Tn*917*ΩHU*13*); 5, BK410 (*spoIIIC94*), 6, BK395 (*spoIIID83*); 7, SC622 (*spoIIIE36*); 8, BK338 (*spoIIIGΔ1*); 9, KS194 (*spoIVA*::Tn*917*ΩHU*194*); 10, strain 67 (*spoIVA67*, *trpC2*); 11, BK750 (*spoIVB*::*ermG*); 12, BK558 (*spoIVCA133*); 13, BK556 (*spoIVCB23*); 14, SC834 (*spoIVF152*); 15, KS301 (*spoIVF*::Tn*917*ΩHU*301*); 16, KS179 (*spoIVF*::Tn*917*ΩHU*179*). These strains are isogenic to PY79 (27), except 131.5 and 67 are isogenic to SG38 (28). These strains have been described (7, 14, 15, 29), except BK750 was constructed by transformation of DNA prepared from JH12719 (30) into PY79 with selection for the erythromycin-resistance gene (*ermG*) inserted in the *spoIVB* gene (B. Kunkel and R. Losick, personal communication) and KS301 has Tn*917* inserted in the *spoIVF* locus (S. Cutting and R. Losick, personal communication). The Tn*917* insertions *HU194* and *HU179* were thought to define new loci designated *spoVP* and *spoVL*, respectively (31), but are now assigned to the indicated loci (8).

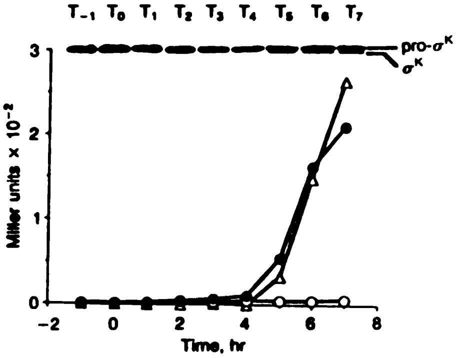

FIG. 5. Effect of producing pro-σ^K from a plasmid during growth and sporulation of *B. subtilis*. Strain BK410 (*spoIIIC94*; ref. 15) was transformed with the P_{spac}–*sigK* fusion plasmid (pSL1) or the control plasmid (pDG148), and both resulting strains were lysogenized with phage SPβ::*cotD–lacZ*, resulting in strains BSL3 and BSL4, respectively. The Spo⁺ strain PY79 (27) was also lysogenized with SPβ::*cotD–lacZ*, resulting in strain BSL5. Cells were grown and sporulated in DS medium with the addition of 1 mM IPTG ~2 hr before the end of exponential growth. Samples were harvested at hourly intervals and β-galactosidase activity was determined (35) using the substrate *o*-nitrophenol β-D-galactoside. One unit of enzyme hydrolyzes 1 μmol of substrate per min per OD_{595} unit of initial cell density. Background activity (ranging from 0.5 to 6 units) of PY79 at each time point was subtracted from the values obtained for strains containing the *cotD–lacZ* fusion. *cotD*-directed β-galactosidase activity was determined for strains BSL3 (●), BSL4 (○), and BSL5 (△). (*Inset*) Western blot analysis of whole-cell extracts (10 μg of protein) of strain BSL3, using the anti-pro-σ^K antibodies.

until T_4 (●). Pro-σ^K produced in *B. subtilis* appears to be inactive as a σ factor, unless the presence or absence of another regulatory factor(s) prevents *cotD* transcription during growth and early in sporulation. Beginning at T_4, and more noticeably at T_5, σ^K was observed by Western blot analysis and *cotD*-directed β-galactosidase activity increased significantly compared to the level observed in a control strain harboring a plasmid without *sigK* (Fig. 5, ○). The increase in *cotD*-directed β-galactosidase activity paralleled that observed from the *cotD–lacZ* fusion in wild-type *B. subtilis* (△). Thus, σ^K was first detected at T_4 and increased through T_6 in wild-type cells (Fig. 3) or in cells expressing pro-σ^K from a plasmid (Fig. 5), and in both cases the increase in the σ^K level coincided with the increase in *cotD*-directed β-galactosidase activity (Fig. 5). The finding that σ^K accumulated at the normal time in cells expressing pro-σ^K from a plasmid during growth and early in sporulation demonstrates that production of pro-σ^K is not the limiting factor in the production of σ^K. This suggests that if σ^K is derived from pro-σ^K by proteolytic processing, the processing step itself may be a developmentally regulated event that begins at about T_4.

DISCUSSION

The primary product of *sigK* was inferred to be a pro-protein (pro-σ^K) with 20 extra residues at the N terminus based on a comparison of the N-terminal amino acid sequence of σ^K (6) with the nucleotide sequence of *sigK* (12). Using anti-pro-σ^K antibodies in Western blot analyses of whole-cell extracts of sporulating *B. subtilis*, we detected proteins that we believe are pro-σ^K and σ^K for the following reasons: (*i*) the proteins comigrated with gel-purified pro-σ^K and σ^K (Fig. 2), (*ii*) the proteins were not observed in Western blot analyses of whole-cell extracts prepared from growing wild-type cells (Fig. 2) or from developing cells of five strains that were expected to be unable to produce pro-σ^K and σ^K due to a mutation either in the *sigK* structural gene or in a gene whose product is required to generate the composite *sigK* gene (Fig. 4), and (*iii*) the protein that comigrated with pro-σ^K was first observed in Western blot analysis of wild-type cells at T_3 (Fig. 3), which is consistent with the timing of *sigK* expression (14), while the protein that comigrated with σ^K was first observed at T_4 (Fig. 3), which is consistent with the timing of expression of the σ^K-controlled *cotA* regulon (7–9).

Proteolytic processing has been shown to control the activity of σ^E (16, 17), another sporulation-specific σ factor in *B. subtilis*. By analogy to σ^E and based on the finding that the N terminus of σ^K corresponds to codon 21 of *sigK*, it was proposed that pro-σ^K may be an inactive precursor that is proteolytically processed to active σ^K (6, 12). Several of our results are consistent with this model. First, the appearance of pro-σ^K preceded the appearance of σ^K during sporulation of wild-type *B. subtilis* (Fig. 3), and the timing of appearance of *cotD*-directed β-galactosidase activity (Fig. 5, △) coincided with the appearance of σ^K, not pro-σ^K. Second, mutations in eight sporulation loci (*spoIIB*, *spoIID*, *spoIIIA*, *spoIIIE*, *spoIIIG*, *spoIVA*, *spoIVB*, and *spoIVF*) blocked or reduced accumulation of σ^K, but not accumulation of pro-σ^K (Fig. 4), and the impaired *cotA* regulon expression in strains with these mutations (7–9) correlates with the impaired accumulation of σ^K, not with the level of pro-σ^K, which was normal in all these mutants except the *spoIIB* mutant (see below). Third, when pro-σ^K was gel-purified from *E. coli* and renatured under the same conditions that permit recovery of activity of σ^K gel-purified from *B. subtilis*, it failed to promote transcription of σ^K-controlled promoters *in vitro* (data not shown). Fourth, production of pro-σ^K from a plasmid in a *B. subtilis sigK* mutant resulted in production of σ^K during sporulation (Fig. 5 *Inset*), and, just as in wild-type cells, the timing of appearance of *cotD*-directed β-galactosidase activity (Fig. 5, ●) coincided with the appearance of σ^K, not with the level of pro-σ^K, which was high during growth and throughout sporulation. Our data do not rule out the interpretation that σ^K is produced by translational initiation at an alternative site; however, this possibility is unlikely since no apparent ribosome-binding site or initiation codon exists at the appropriate position in the *sigK* mRNA. Nevertheless, it may be possible to use a pulse–chase experiment to demonstrate directly a precursor–product relationship between pro-σ^K and σ^K, as has been done in the case of σ^E and its precursor (17). Proof that pro-σ^K is an inactive precursor that can be proteolytically processed to active σ^K will require reconstitution of the processing reaction *in vitro*.

The accumulation of σ^K is a developmentally regulated event that begins at about T_4 in wild-type cells (Fig. 3) or in *sigK* mutant cells expressing pro-σ^K from a plasmid (Fig. 5). This event directly or indirectly requires proper functioning of the products of at least eight sporulation loci since, as noted above, mutations in eight loci blocked or reduced accumulation of σ^K but not accumulation of pro-σ^K. If σ^K is derived from an inactive precursor by a developmentally regulated proteolytic processing event, what purpose might this regulatory device serve? In the case of σ^E, processing has been suggested to be a mechanism for coupling formation of the sporulation septum to activation of σ^E and the subsequent pattern of gene expression (17, 18). Our finding that *spoIIIA*, *spoIIIE*, *spoIIIG*, and *spoIVB* mutants accumulate pro-σ^K, but not σ^K, and the results of Cutting *et al.* (29), discussed below, suggest that pro-σ^K processing may couple activation of the mother-cell σ factor to events occurring in the forespore compartment.

A regulatory mechanism connecting mother-cell-specific gene expression to forespore events was inferred (7–9, 14) from the observation that mutations in *spoIIIA*, *spoIIIE*, and *spoIIIG* that impair forespore-specific gene expression (32, 33) also impair mother-cell-specific gene expression. Although little is known about the functions of the *spoIIIA* and *spoIIIE* gene products, *spoIIIG* is expressed predominantly, if not exclusively, in the forespore compartment and it encodes a σ factor, σ^G, that directs forespore-specific gene expression (4, 5). Recently, *spoIVB* (30) has been shown to be expressed specifically in the forespore, yet mutations in this gene impair mother-cell-specific gene expression (S. Cutting and R. Losick, personal communication). Cutting *et al.* (29) isolated mutants (called *bof* mutants for *b*ypass *o*f *f*orespore) that bypass the dependence of *cotA* regulon expression on *spoIIIA*, *spoIIIE*, *spoIIIG*, and *spoIVB* mutations. Using the anti-pro-σ^K antibodies described here, it was shown that *bof* mutations restore production of σ^K in *spoIIIA* and *spoIIIG* mutant cells (29). Thus, *bof* mutations appear to uncouple mother-cell-specific gene expression from forespore events by permitting pro-σ^K processing. Furthermore, replacement of *sigK* with a deletion-mutated version lacking codons 2–20 (so that the protein produced, $\sigma^{K\Delta 19}$, would differ from σ^K only by a methionine residue at its N terminus) relieved the dependence of *cotA* regulon expression on the *spoIIIG* gene product (29). In this case the proposed coupling between forespore events and pro-σ^K processing appears to be circumvented by producing the truncated, active $\sigma^{K\Delta 19}$ instead of pro-σ^K. A protein that was presumably $\sigma^{K\Delta 19}$, since it comigrated with σ^K in Western blot analysis using the anti-pro-σ^K antibodies, was detected beginning at T_3 in a *spoIIIG* mutant containing the deletion-mutated *sigK* gene (data not shown). This finding suggests that the failure of the *spoIIIG* mutant to accumulate σ^K when it contains an intact *sigK* gene (Fig. 4, lane 8) results from a failure to process pro-σ^K rather than from instability of σ^K, unless $\sigma^{K\Delta 19}$ is significantly more stable than σ^K in the *spoIIIG* mutant. Cells containing the deletion-mutated *sigK* gene also began expressing a *cotA–lacZ* fusion at T_3, 1 hr earlier than normal (29), as would be expected if $\sigma^{K\Delta 19}$ but not pro-σ^K were able to function as a σ factor. The results presented here and the results of Cutting *et al.* (29) strongly suggest that pro-σ^K processing is a regulatory device that couples mother-cell gene expression to forespore morphogenesis. The *spoIIIA*, *spoIIIE*, *spoIIIG*, and *spoIVB* mutations are inferred to block forespore morphogenesis at a stage that is incompatible with pro-σ^K processing.

Mutations in the *spoIIB*, *spoIID*, and *spoIVF* loci also blocked accumulation of σ^K, but not pro-σ^K (Fig. 4). The *spoIIB* mutant used in this study was shown previously to express only 6% of the β-galactosidase activity normally expressed from a *sigK–lacZ* fusion during sporulation (14). This may explain the reduced amount of pro-σ^K detected by the anti-pro-σ^K antibodies in this mutant (Fig. 4, lane 1). Production of pro-σ^K was unimpaired in the *spoIID* mutant (Fig. 4, lane 2). Since *spoIID* mutations have been shown to impair forespore-specific gene expression (5, 32, 33), perhaps these mutations also block forespore morphogenesis at a stage that is incompatible with pro-σ^K processing. The *spoIVF* locus is the best candidate to encode a protein directly involved in the pro-σ^K processing reaction. Mutations in *spoIVF* block expression of the *cotA* regulon (7–9) and our results show that these mutants accumulate pro-σ^K but not σ^K (Fig. 4, lanes 14–16). Like a *spoIIIG* mutant, a *spoIVF* mutant engineered to produce truncated, active $\sigma^{K\Delta 19}$ expresses the *cotA* gene (29). However, a *spoIVF* mutation does not block the expression of a forespore-specific gene (32). Furthermore, a *bofA* mutation does not bypass the dependence of *cotA* expression on a *spoIVF* mutation, and the *bofB* mutations are alleles of the *spoIVF* locus (29). These results have led to the proposal that the *spoIVF* gene product(s) governs processing of pro-σ^K to σ^K and that *bofB* mutations alter the *spoIVF* gene product(s) so as to relieve its dependence on the products of *spoIIIA*, *spoIIIE*, *spoIIIG*, and *spoIVB* (29).

As far as we know, σ^E (17, 18) and σ^K are the only transcription factors thought to be synthesized as inactive precursor proteins and activated by specific proteolytic cleavages. In both cases, proteolytic processing may couple completion of a morphogenetic step to the subsequent, new pattern of gene expression, but in each case the molecular mechanism of the coupling remains to be elucidated.

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APPENDIX B

Sporulation Regulatory Protein GerE from *Bacillus subtilis* Binds To and Can Activate or Repress Transcription from Promoters for Mother-Cell-Specific Genes

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Sporulation Regulatory Protein GerE from *Bacillus subtilis* Binds to and Can Activate or Repress Transcription from Promoters for Mother-cell-specific Genes

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The mother-cell line of gene expression during sporulation in *Bacillus subtilis* is a hierarchical cascade consisting of at least four temporally controlled gene sets, the first three of which each contain a regulatory gene for the next gene set in the pathway. *gerE*, a member of the penultimate gene set, is a regulatory gene whose product is required for the transcriptional activation of genes (coat protein genes *cotB* and *cotC*) in the last gene set. The *gerE* product also influences the expression of other members of the penultimate gene set (coat protein genes *cotA* and *cotD* appear to be repressed and activated, respectively). We now report that the purified product of *gerE* (GerE) is a DNA-binding protein that adheres to the promoters for *cotB* and *cotC*. We also show that GerE stimulates *cotB* and *cotC* transcription *in vitro* by RNA polymerase containing the mother-cell sigma factor σ^K. These findings support the view that GerE is a positively acting, regulatory protein whose appearance at a late stage of development directly activates the transcription of genes in the last known temporal class of mother-cell-expressed genes. In addition, GerE stimulates *cotD* transcription and inhibits *cotA* transcription *in vitro* by σ^K RNA polymerase, as expected from *in vivo* studies, and, unexpectedly, profoundly inhibits *in vitro* transcription of the gene (*sigK*) that encodes σ^K. The effects of GerE on *cotD* and *sigK* transcription are just the opposite of the effects exerted by the earlier-appearing, mother-cell regulatory protein SpoIIID, suggesting that the ordered appearance of first SpoIIID, then GerE, ensures proper flow of the regulatory cascade controlling gene expression in the mother cell.

Keywords: sporulation; sigma factor; regulatory protein; *Bacillus subtilis*

1. Introduction

Following the formation of a transverse septum at morphological stage II, gene expression during the process of sporulation in *Bacillus subtilis* is regulated differentially between the forespore and mother-cell chambers of the developing sporangium (De Lencastre & Piggot, 1979; Losick & Stragier, 1992). Thus, the transcription of certain genes is restricted to the forespore, whereas the transcription of other genes is limited to the mother cell. Although some heterogeneity in the time of induction of gene expression in the forespore has been reported (Panzer *et al.*, 1989; Sussman & Setlow, 1991), most forespore-expressed genes are switched on co-ordinately and only a single regulatory gene, *spoIIIG*, which encodes the forespore sigma factor σ^G (Karmazyn-Campelli *et al.*, 1989; Sun *et al.*, 1989), is known to be exclusively transcribed in the forespore chamber of the sporangium. Gene expression in the mother cell, in contrast, is relatively complex, involving the expression of at least four co-ordinately controlled gene sets, which are switched on successively during the course of sporulation (Cutting *et al.*, 1989; Kunkel *et al.*, 1988, 1989; Sandman *et al.*, 1988; Zheng & Losick, 1990).

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These gene sets constitute a hierarchical cascade in that the first three gene sets each contain a regulatory gene that governs the expression of the next gene set in the pathway (Zheng & Losick, 1990). *spoIIID*, a member of the earliest regulon, is a regulatory gene whose product (a small, DNA-binding protein; R.H. & L.K, unpublished results) turns on the transcription of the next set of genes (Halberg & Kroos, 1992; Kroos *et al.*, 1989; Kunkel *et al.*, 1989; Stevens & Errington, 1990). One of the genes in the *spoIIID*-dependent class of coordinately controlled genes is *sigK* (Kroos *et al.*, 1989; Kunkel *et al.*, 1988, 1989), a composite gene (generated from two truncated genes by a DNA rearrangement in the mother-cell chromosome; Kunkel *et al.*, 1990; Stragier *et al.*, 1989) that encodes the mother-cell sigma factor σ^K (Kroos *et al.*, 1989). The *sigK* gene product (after a regulatory step involving the conversion of its primary product, pro-σ^K, to the mature sigma factor; Cutting *et al.*, 1990; Lu *et al.*, 1990) then turns on the penultimate class of genes. This set of genes includes the spore coat protein genes *cotA* and *cotD* (Sandman *et al.*, 1988; Zheng & Losick, 1990) and the regulatory gene *gerE* (Cutting *et al.*, 1989; Holland *et al.*, 1987), which encodes a product (GerE) that is, in turn, required for the expression of genes in the last known gene set in the mother-cell line of gene expression (Zheng & Losick, 1990). Two members of this *gerE*-dependent gene set are the coat protein genes *cotB* and *cotC* (Donovan *et al.*, 1987).

Here we are concerned with the role of *gerE* in the hierarchical cascade of mother-cell gene expression. *gerE* is inferred to be a transcriptional regulatory gene because: (1) a *gerE* nonsense mutation (*gerE36*; Cutting, 1986) has highly pleiotropic effects on sporulation, causing the production of spores that are lysozyme-sensitive, germination-defective and aberrant in coat ultrastructure and protein composition (Feng & Aronson, 1986; Jenkinson & Lord, 1983; Moir, 1981); (2) *gerE36* partially inhibits expression of *cotD* (Zheng & Losick, 1990) and causes overexpression of *cotA* in rich sporulation medium (Cutting *et al.*, 1989; Sandman *et al.*, 1988); (3) as indicated above, *gerE36* prevents the expression of cot genes *B* and *C* (Zheng & Losick, 1990); and (4) the predicted product of *gerE* (GerE), an 8·5 kDa polypeptide (Cutting & Mandelstam, 1986; Hasnain *et al.*, 1985), contains a region of similarity to the α-helix–β-turn–α-helix motif of many procaryotic transcriptional regulatory proteins (Holland *et al.*, 1987) and exhibits high overall similarity to the COOH-terminal region of certain regulatory members (the BvgA sub-group) of the family of two-component sensor-regulator systems in bacteria (Gross *et al.*, 1989; Kahn & Ditta, 1991), which includes the *B. subtilis* regulatory proteins DegU and ComA (Henner *et al.*, 1988; Kunst *et al.*, 1988; Weinrauch *et al.*, 1989). GerE is also similar to the COOH-terminal region of the *Escherichia coli* regulatory protein MalT (Gross *et al.*, 1989) and to the COOH-terminal region of sigma factors, which is involved in the recognition of the −35 region of bacterial promoters (Kahn & Ditta, 1991).

On the basis of DNase I footprinting experiments with purified GerE, we now report that the *gerE* gene product is a DNA-binding protein that adheres to the regulatory regions for *cotB* and *cotC*. We also show that GerE greatly stimulates *cotB* and *cotC* transcription *in vitro* by σ^K RNA polymerase, a finding in support of the view that the appearance of GerE at a late stage of sporulation directly activates transcription of these genes. Moreover, we show that GerE affects the *in vitro* transcription of several other mother-cell-expressed genes, either positively or negatively, findings that suggest a functional analogy between GerE and the mother-cell regulatory protein SpoIIID (Kroos *et al.*, 1989). However, the effects of GerE on *cotD* and *sigK* transcription *in vitro* are just the opposite of the effects exerted by SpoIIID. Because production of σ^K RNA polymerase appears to cause a decrease in the level of SpoIIID (Halberg & Kroos, 1992) and is required for the transcription of *gerE* (Cutting *et al.*, 1989; and this work), we propose that a declining level of SpoIIID and a rising level of GerE produce a reinforced switch in the pattern of mother-cell gene expression during sporulation.

2. Materials and Methods

(a) *Strains and plasmids*

E. coli K38 (HfrC *trp thi* λ^+) carrying plasmid pGP1-2 (Tabor & Richardson, 1985) was maintained at 30°C in LB medium containing 25 μg of kanamycin/ml.

The source of the *gerE* open reading frame was pSGMU101 (provided by J. Errington of Oxford University; Cutting & Mandelstam, 1986). The *gerE* open reading frame was released as a 0·5 kb† *Eco*RV/*Xba*I fragment and was cloned into the *Sma*I/*Xba*I site of pT713 (Bethesda Research Laboratories) to create pLZ304. The *Eco*RV site is 67 bp upstream from the *gerE* coding sequence, whereas the *Xba*I site is a polylinker site adjacent to a *B. subtilis Mbo*I site located 227 bp downstream from the *gerE* open reading frame.

Plasmids pLZ304 and pT713 were introduced into K38 cells containing pGP1-2 by transformation and selection on LB medium containing 50 μg of ampicillin/ml and 25 μg of kanamycin/ml at the permissive temperature (30°C).

Plasmids pLRK100 and pBK16 containing the *cotD* and *sigK* promoter regions, respectively, served as templates for *in vitro* transcription and have been described previously (Kroos *et al.*, 1989). A 0·6 kb *Eco*RI/*Pvu*II fragment (Fig. 1) containing the *cotB* promoter region (Zheng & Losick, 1990) was subcloned into *Eco*RI/*Sma*I-digested replicative form M13mp18 (Yanisch-Perron *et al.*, 1985) and replicative form DNA of the recombinant phage was used for *in vitro* transcription. Plasmid pBD261 containing the *cotC* promoter region was constructed as follows: the *lacZ-cat*-containing *Bam*HI fragment of pSGMU31 (Errington, 1986) was cloned into the *Bcl*I site of pBD95 (Zheng & Losick, 1990), creating an in-frame fusion of *cotC* to *lacZ* in pBD239, then the

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†Abbreviations used: kb, 10^3 bases or base-pairs; bp, base-pairs; nt, nucleotides.

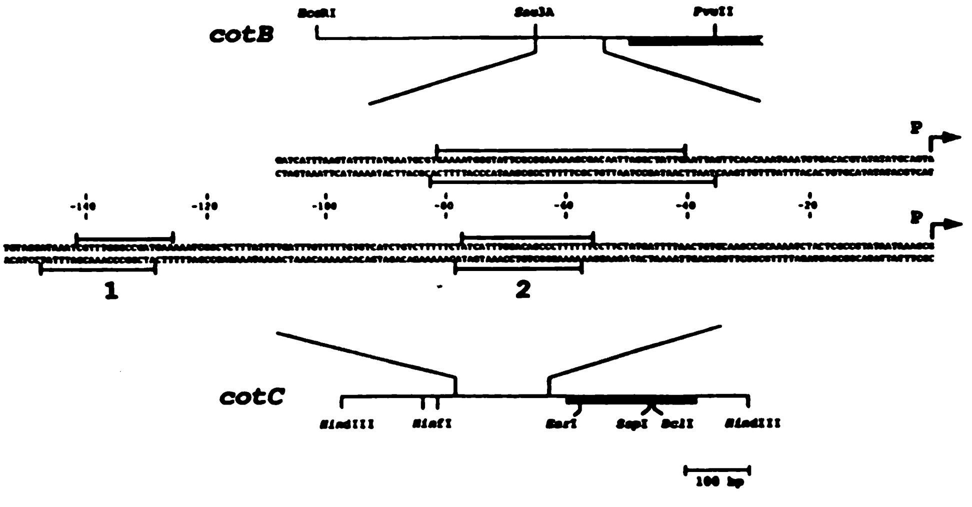

Figure 1. The GerE binding sites in the 5' regions of *cotB* and *cotC*. The Figure shows restriction maps of DNA in the vicinity of *cotB* and *cotC*, based on a previous report (Donovan *et al.*, 1987) and other unpublished analysis. (Note that the map of *Sau*3A sites is incomplete and only a single site is shown.) The positions of the open reading frames for each gene are shown by the filled bars (the map includes only part of the *cotB* open reading frame). Also shown are the nucleotide sequences of the promoter regions of both genes. The startsites of transcription are indicated by the arrows. Regions of DNA that were protected by GerE from the action of DNase I are indicated above and below each strand. The 2 GerE binding sites in *cotC* are designated 1 and 2 in the Figure.

cotC-lacZ-cat-containing *Bam*HI/*Bgl*II fragment of pBD239 was cloned into the *Bam*HI site of pBR322 (Bolivar *et al.*, 1977) to construct pBD259 in which the *Hind*III site upstream from the *cotC* promoter is proximal to the *Hind*III site of the vector, and finally pBD259 was digested with *Hind*III and recircularized to construct pBD261 in which the small *Hind*III fragment was deleted. pHI1 was constructed by subcloning a 0·6 kb *Hind*III/*Xba*I fragment from pBD261 (extending from the *Hind*III site upstream from *cotC* to an *Xba*I site in the polylinker downstream from the former *Bcl*I site of *cotC* (Fig. 1) into *Hind*III/*Xba*I-digested pUC19 (Yanisch-Perron *et al.*, 1985). Restriction fragments from pHI1 were purified after electrophoresis on an agarose gel and used as templates for *in vitro* transcription of *cotC*. The *gerE* promoter-containing plasmid, pSC146, was constructed by S. Cutting as follows: the 266 bp *Alu*I fragment from pSGMU101 (encompassing the *gerE* promoter region; Cutting & Mandelstam, 1986) was subcloned into the *Sma*I site in the polylinker of pSGMU31 (Errington, 1986), then excised as a *Kpn*I/*Bam*HI fragment and inserted into *Kpn*I/*Bam*HI-digested pUC19 (Yanisch-Perron *et al.*, 1985). Three *cotA* promoter-containing plasmids were constructed by K. Sandman as follows: (1) pKS22 was constructed by ligating *Hind*III linkers to a 0·8 kb *Hinc*II/*Ava*I fragment from pKS11 (encompassing the *cotA* promoter region; Sandman *et al.*, 1988), cleaving the linkers with *Hind*III, and subcloning the fragment into *Hind*III-digested pIBI76 (International Biotechnologies, Inc.); (2) pKS23 was constructed by digesting pKS19 (Sandman *et al.*, 1988) with *Pst*I and ligating to delete *B. subtilis* DNA beyond 55 bp upstream from the *cotA* transcriptional startsite; (3) pKS24 was constructed by digesting pKS19 (Sandman *et al.*, 1988) with *Eco*RV and ligating to delete *B. subtilis* DNA beyond 115 bp upstream from the *cotA* transcriptional startsite.

(b) *Production of GerE in* E. coli

Cultures of K38 cells containing pGP1-2 (bearing the phage T7 RNA polymerase gene) alone, pGP1-2 and pT713, or pGP1-2 and pLZ304 were grown at 30°C to an A_{600} of 0·3. Cells were induced by a temperature shift to 42°C for 20 min. Rifampicin was added to a final concentration of 200 μg/ml, and the cells were incubated at 42°C for 10 min and then at 30°C for 30 min. Cells were collected by centrifugation and dissolved in sample buffer (Laemmli, 1970). The sample was denatured at 90°C for 2 min and fractionated by electrophoresis through an SDS/polyacrylamide gel containing 15% acrylamide.

For the preparation of GerE, protein from induced K38 cells containing pGP1-2 and pLZ304 was subjected to electrophoresis and the putative GerE band was cut from the gel. GerE was then eluted from the gel slice and renatured as described (Hager & Burgess, 1980). For the preparation of control protein, protein from induced K38 cells containing pGP1-2 and pT713 was subjected to electrophoresis. A slice from the position corresponding to that of GerE was cut from the gel. Protein was then eluted and renatured from the gel slice as described for GerE.

(c) *Preparation of DNA probes labeled at only one end*

For the preparation of radioactive *cotC* probes, a 358 bp *Hinf*I/*Bcl*I fragment (Fig. 1) whose *Hinf*I terminus had been rendered flush by use of the Klenow fragment of DNA polymerase I was cloned into *Hinc*II/*Bam*HI-digested pUC18 (Yanisch-Perron *et al.*, 1985). This created plasmid pLZ1275 in which the *Hin*dIII site of the vector was upstream from the *cotC* promoter. Plasmid pLZ1275 was linearized with *Hin*dIII and then treated with alkaline phosphatase. Next, the *cotC*-containing fragment was released from the pUC18 vector by digestion with *Eco*RI, which cuts at the end of the polylinker next to the *Bam*HI site. A probe labeled at the *Hin*dIII site in the non-transcribed strand was prepared using phage T4 polynucleotide kinase and [γ-^{32}P]ATP. To prepare a probe labeled at the *Hin*dIII site in the transcribed strand, *cotC* was released as a *Hin*dIII/*Sma*I fragment (*Sma*I also cuts in the polylinker next to *Bam*HI site) and was labeled by end-filling the *Hin*dIII terminus using the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP. Additional *cotC* probes were prepared by digesting pHI1 (see above) with *Ear*I, which cleaves in the 7th codon of *cotC* (Donovan *et al.*, 1987), labeling in the non-transcribed strand using the fill-in reaction of the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP or labeling in the transcribed strand by treatment with alkaline phosphatase followed by phage T4 polynucleotide kinase and [γ-^{32}P]ATP, then digesting with *Eco*RV and *Eco*RI, and purifying the 239 bp *Eco*RV/*Ear*I fragment encompassing the *cotC* promoter region after electrophoresis on a non-denaturing, polyacrylamide gel containing 8% acrylamide (Maniatis *et al.*, 1982).

For preparation of radioactive *cotB* probes, we took advantage of a plasmid pUC18 derivative called pBD136 (constructed by W. Donovan, unpublished results), which contains a 0·8 kb *Sau*3AI/*Sau*3AI fragment that includes the promoter and the NH_2-terminal coding region of the *cotB* open reading frame cloned into the *Bam*HI site in an orientation such that the polylinker *Eco*RI site was proximal to and upstream from the promoter. pBD136 was digested with *Eco*RI, treated with alkaline phosphatase, and the *cotB*-containing fragment was released by digestion with *Hin*dIII, which cuts at the opposite end of the polylinker. The non-transcribed strand probe was labeled at the *Eco*RI site by the T4 polynucleotide kinase reaction. To prepare the transcribed strand probe, a *cotB*-containing, *Eco*RI/*Pvu*II fragment was purified (see Fig. 1) and was then labeled by end-filling using the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP.

(d) *DNase I footprinting*

Two different methods were used to carry out the DNase I footprinting experiments. In method (1), the conditions for the binding of GerE were the same as described by Strauch *et al.* (1989), except that poly(dI-dC) was added to a final concentration of 30 μg/ml. DNA fragments labeled at one end were incubated in separate experiments without protein, with control protein, or with different amounts of GerE protein in 20 μl reaction mixtures for 15 min. Then 1 μl of a 0·01 mg/ml DNase I solution (BRL) was added to each reaction for 1 min. The digests were stopped by adding 5 μl of stop solution (0·1 M-EDTA and 0·5% SDS) and chilled on ice. The DNA in each reaction was precipitated with 1 ml of ethanol and with 0·2 μg of poly(dI-dC) as carrier. The precipitates were dissolved in formamide loading buffer (Maniatis *et al.*, 1982) and denatured at 90°C for 90 s. The samples were then subjected to electrophoresis in an 8 M-urea-containing polyacrylamide gel.

In method (2) the conditions for the binding of GerE were the same as in the *in vitro* transcription experiments (see below), except that poly(dI-dC) was added to a final concentration of 1·2 μg/ml. DNA fragments labeled at one end were incubated at 37°C in separate experiments without protein, with control protein, or with different amounts of GerE in 42 μl reaction mixtures for 10 min. Then 3 μl of 0·0004 mg DNase I/ml (Boehringer-Mannheim) solution (prepared by diluting a stock solution (Davis *et al.*, 1980) with buffer (20 mM-Tris·HCl, pH 8·0, 20 mM-$MgCl_2$, 20 mM-$CaCl_2$) was added to each reaction. After 1 min, the digests were stopped by adding 50 μl of buffer (100 mM-Tris·HCl, pH 8·0, 50 mM-EDTA, 200 μg yeast tRNA/ml) and incubating for 2 min at 65°C. The DNA in each reaction was precipitated with 250 μl ethanol. The precipitates were dissolved in formamide loading buffer (Maniatis *et al.*, 1982) and denatured by boiling. The samples were then subjected to electrophoresis in an 8 M-urea/polyacrylamide gel containing 6% acrylamide.

(e) *DNA sequencing*

End-labeled DNA probes were subjected to the chemical cleavage reactions of Maxam & Gilbert (1980) with a kit from New England Nuclear or as described previously (Maniatis *et al.*, 1982).

(f) In vitro *transcription*

σ^K RNA polymerase was partially purified from *B. subtilis* strain SC104 (*trpC2 gerE36* SPβ::*cotA-lacZ*) as described (Kroos *et al.*, 1989). This enzyme was comparable in protein composition and in *cotD*- and *sigK*-transcribing activities to fraction 24 shown in Fig. 2 of Kroos *et al.* (1989). σ^K RNA polymerase was reconstituted from gel-purified, renatured σ^K and *B. subtilis* core RNA polymerase as described previously (Kroos *et al.*, 1989). Transcription reactions were performed as described previously (Kroos *et al.*, 1989) except that heparin (6 μg) was added 2 min after the addition of nucleotides to prevent reinitiation, and after the reactions were stopped 10 μl of the reaction mixture was subjected to electrophoresis. [α-^{32}P]CTP was the labeled nucleotide unless indicated otherwise. After gel electrophoresis, transcripts were detected by autoradiography and the signals were quantitated using a Visage 110 Image Analyzer (BioImage).

(g) *Primer extension analysis*

RNA was prepared from sporulating cells as described by Cutting *et al.* (1991*a*). *In vitro* synthesized *cotC* transcripts were generated as described above (but without radiolabeled nucleotide) and then precipitated with ethanol and suspended in 25 μl of diethylpyrocarbonate-treated water. In preparation for primer extension analysis, a sample of the *in vitro* synthesized RNA (4 μl) was treated with 5 units of DNase I (Pharmacia) in buffer (20 mM-Tris·HCl, pH 7·6, 10 mM-$MgCl_2$) in a total reaction volume of 5 μl. The reaction was incubated at 37°C for 10 min and then at 90°C for 10 min.

Primer extension was carried out by use of the *cotC*-specific oligonucleotides Pr1 and Pr2 (Zheng & Losick, 1990). The oligonucleotides were 5′-end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (BRL) as described by Sambrook *et al.* (1989). For analysis of *in vivo* synthesized RNA, from 2 to 6 pmol of 5′-end labeled oligonucleotide was incubated with 5 μg of total RNA, and reactions were carried out as described by Roels *et al.* (1992). For analysis of *in vitro*-generated *cotC* transcripts, 12 pmol of 5′-end-labeled Pr2 oligonucleotide was incubated with 4 μl *in vitro* synthesized RNA (from above) in 10 μl of annealing buffer (50 mM-Tris·HCl, pH 7·6, 100 mM-KCl) at 90°C for 2 min and then at 47°C for

30 min; 5 μl of the primer:RNA hybrid solution was then incubated with 5 units of phage M-MuLV reverse transcriptase (Pharmacia) at 47 °C for 45 min in a final volume of 10 μl of reverse transcriptase buffer (50 mM-Tris·HCl, pH 7·6, 60 mM-KCl, 10 mM-$MgCl_2$, 1 mM-dithiothreitol (DTT), 2·5 mM of each dNTP, 1 unit placental RNase inhibitor/μl (Pharmacia)) after which 7 μl of 95% formamide loading dye was added.

The 5′-end-labeled oligonucleotides were also used to generate sequence ladders by the dideoxy chain termination method of Sanger *et al.* (1977). The products of primer extension were subjected to electrophoresis in 6% polyacrylamide slab gels containing 8 M-urea.

3. Results

(a) *Purification of GerE*

GerE was purified by engineering *E. coli* cells to express a cloned copy of the *gerE* gene using the T7 RNA polymerase/T7 promoter system of Tabor & Richardson (1985). A DNA fragment containing the *gerE* open reading frame was placed under the control of a phage T7 promoter by inserting into the expression vector pT713 a *gerE*-containing segment of *B. subtilis* DNA that extended 67 bp upstream and 227 bp downstream from the *gerE* open reading frame to create plasmid pLZ304 (see Materials and Methods). Cells containing pLZ304, the vector pT713, or neither plasmid, were grown at 30°C and were then shifted to 42°C to induce transcription from the phage T7 promoter. Total cellular proteins from induced and uninduced cells were displayed by electrophoresis through an SDS/polyacrylamide gel (Fig. 2). In addition to normal cellular proteins, induced cells of the pLZ304-containing strain (lane F) produced a protein of 6 to 8 kDa, which was absent in induced cells that lacked the *gerE*-bearing plasmid (lane D) or that contained the plasmid vector, pT713 (lane E). This protein was also absent in cells that were not heat-induced (lanes A to C). The size of the protein was consistent with that (8·5 kDa) deduced from the nucleotide sequence of the *gerE* open reading frame (Cutting & Mandelstam, 1986). Because there was no other open reading frame in the *gerE*-bearing *B. subtilis* insert in pLZ304 that could encode a protein of this size, we presume that the 6 to 8 kDa protein was GerE.

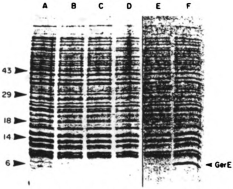

Figure 2. Production of GerE in *E. coli*. Total cellular proteins were extracted from cells grown at 30°C (lanes A to C) or from cells that had been shifted to 42°C (lanes D to F) and were then resolved by electrophoresis in a SDS/12% polyacrylamide gel. The cells were derivatives of *E. coli* strain K38 containing pGP1-2 alone (lane A and D), pGP1-2 and pT713 (lane B and E), or pGP1-2 and pLZ304 (lane C and F). (pGP1-2 contains the phage T7 RNA polymerase gene.) The positions of the molecular mass markers (in kDa) are shown on the left.

To purify GerE, the 6 to 8 kDa protein band was excised from an SDS/polyacrylamide gel displaying proteins from pLZ304-containing bacteria. Protein was eluted from the gel slice and renatured as described by Hager & Burgess (1980). As a control, a gel slice corresponding in position to GerE was cut from an SDS/polyacrylamide gel displaying total cellular proteins from induced cells of pT713-containing bacteria. Protein was eluted from the gel slice and renatured and is referred to as "control protein".

(b) *Mapping the 5′ terminus of* cotC *mRNA*

To study the interaction of GerE with *cotB* and *cotC*, it was first necessary to know the precise sites from which transcripts from these genes originate. Previously reported primer extension experiments established the location of the 5′ terminus of *cotB* mRNA (see Fig. 1) but only provided a tentative assignment for the 5′ terminus of *cotC* mRNA (Zheng & Losick, 1990); an extension product of 127 nt that would correspond to an apparent 5′ terminus located 120 bp upstream from the *cotC* initiation codon was obtained with an oligonucleotide primer called Pr1 but no extension products were observed with two other oligonucleotides called Pr2 and Pr3 (see Fig. 1 of Zheng & Losick (1990) for a description of the primers). Alerted from the results of *in vitro* transcription experiments (below) that the true startsite of transcription was actually very close to the beginning of the *cotC* open reading frame (and hence that relatively short extension products were to be expected), we repeated the primer extension analysis and found that Pr1 and Pr2 generated extension products of 33 and 68 nt, respectively, that corresponded (as judged by electrophoresis alongside Pr1 and Pr2-generated nucleotide sequencing ladders; Fig. 3(a) and 3(b)) with a 5′ terminus that preceded the initiation codon by only 26 bp (Fig. 1). These extension products were specific to $gerE^+$ cells at a late stage of sporulation in that Pr1 and Pr2 generated little or no extension products with RNA from wild-type cells at an early stage of development or with RNA from *gerE* mutant cells at a late stage of development (Fig. 3(d)). Consistent with our earlier results (Zheng & Losick, 1990), Pr1 generated the previously observed 127 nt extension product in addition to the 33 nt product (Fig. 3(a)), but direct nucleotide sequencing of this extension product by the use of dideoxynucleotides in the primer exten-

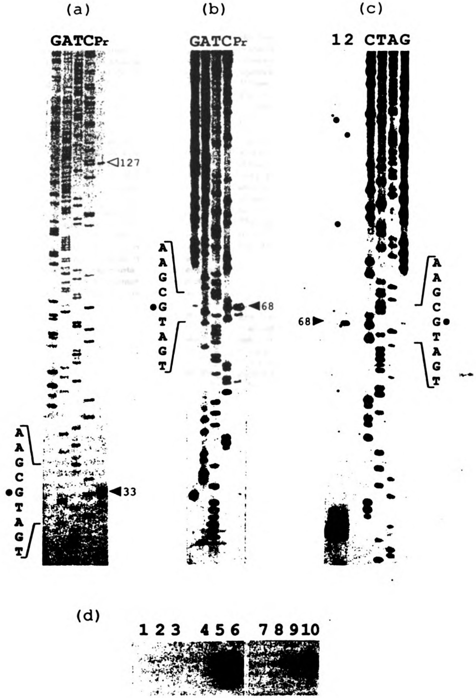
(a)
GATCPr
127
A
A
G
C
G
T
A
G
T
33
(b)
GATCPr
A
A
G
C
G
T
A
G
T
68
(c)
1 2 CTAG
68
A
A
G
C
G
T
A
G
T
(d)
1 2 3 4 5 6 7 8 9 10

Fig. 3.

sion reaction established that the 127 nt product was not copied from *cotC* mRNA but rather from the transcript of another, similarly regulated gene (data not shown). Finally, the previous failure to observe an extension product with Pr3 is now explained by the fact that this primer corresponds to a sequence (see Fig. 1 of Zheng & Losick, 1990) that is located upstream from the 5′ terminus of *cotC* mRNA.

(c) *GerE binds to specific sequences*

Preliminary gel retardation experiments indicated that GerE binds to a *Hin*dIII/*Ssp*I fragment of 500 bp containing the 5′ region of *cotC* and to an *Eco*RI/*Pvu*II fragment of 635 bp containing the 5′ region of *cotB* (Fig. 1; and Zheng, 1990). To localize the binding of GerE to *cotB* and *cotC* more precisely, DNase I protection experiments were carried out with radioactive DNA probes separately end-labeled on one or the other DNA strand. The radioactively labeled DNAs were incubated with GerE and then mildly treated with DNase I to generate a spectrum of fragments. After the enzyme digestion step, the DNA fragments were fractionated by gel electrophoresis. Figure 4(a) displays the pattern of fragments generated by enzyme treatment of GerE-bound *cotC* DNA that had been labeled on the non-transcribed strand (the upper strand in Fig. 1) at a *Hin*dIII site located upstream from the promoter. The Figure shows that GerE caused protection from the action of DNase I along an approximately 16 bp stretch of DNA extending from position −126 to position −141 relative to the 5′ terminus of *cotC* RNA. No protection was observed with control protein (lane 1). Likewise, Figure 4(b) shows that GerE protected a 19 bp stretch of DNA extending from position −129 to position −147 on the transcribed strand (the lower strand in Fig. 1) of *cotC* and that no protection was observed with control protein.

A second GerE binding site was mapped in the *cotC* promoter region using DNA probes labeled at the *Ear*I site located downstream from the promoter. Figures 4(c) and (d) show that GerE protected an approximately 22 bp stretch of DNA extending from position −56 to position −77 on the non-transcribed strand (the upper strand in Fig. 1) and an approximately 21 bp stretch of DNA extending from position −58 to position −78 on the transcribed strand (the lower strand in Fig. 1), respectively. In both cases, no protection was observed with control protein. The regions upstream from *cotC* that were protected from DNase I digestion by GerE binding are indicated in Figure 1.

Analogous experiments showed that GerE protected a wide region of *cotB* from DNase I digestion. Figure 4(e) shows that the GerE-protected region on the non-transcribed strand (the upper strand in Fig. 1) was 40 to 50 bp in length, with the strongest protection occurring between position −41 and position −81. Figure 4(f) shows that a similar region was protected on the transcribed strand, the protected region being 47 bp in length and extending from position −36 to position −82. The region upstream from *cotB* that was protected from DNase I digestion by the binding of GerE is indicated in Figure 1.

(d) *GerE stimulates* cotB *and* cotC *transcription* in vitro

To test for effects of GerE on transcription of *cotB* and *cotC in vitro*, linearized DNA templates were transcribed in the presence of GerE or control protein with σ^K RNA polymerase partially purified from a *gerE* mutant (see Materials and Methods). σ^K RNA polymerase produced run-off transcripts of the expected sizes from *cotB* in the presence of GerE (Fig. 5(a), lanes 3 and 4). The signal was sevenfold weaker in the presence of control protein (Fig. 5(a), lane 2) or with no addition (Fig. 5(a), lane 1). σ^K RNA polymerase reconstituted from gel-purified σ^K and purified *B. subtilis* core RNA polymerase was also stimulated by GerE to transcribe *cotB* (data not shown).

Partially purified σ^K RNA polymerase failed to transcribe a linearized plasmid (pBD261) bearing the *cotC* promoter, even in the presence of GerE, apparently because sequences located in the vector portion of the plasmid compete with the sequences located upstream from *cotC* for binding to GerE (data not shown). However, when a 0·5 kb restric-

Figure 3. Mapping the 5′ terminus of *cotC* mRNA. (a) and (b) Results of high-resolution mapping of the 5′ terminus of *cotC* mRNA using the oligonucleotide primers Pr1 (a) and Pr2 (b) to prime cDNA synthesis from total RNA from PY79 (spo^+) cells harvested 10 h after the onset of sporulation (t_{10}). (c) Results of high resolution mapping of the 5′ terminus of *cotC* mRNA using the oligonucleotide primer Pr2 to prime cDNA synthesis from RNA transcribed *in vitro* with σ^K-polymerase in the absence (lane 1) or presence (lane 2) of GerE protein. The products of primer extension were subjected to electrophoresis alongside nucleotide sequencing ladders generated with either the Pr1 or Pr2 primer. The arrows indicate the position and size of the principal extension product(s) obtained with each oligonucleotide primer. The open arrow in (a) indicates the position of an artifact band, seen with Pr1 but not Pr2, that is not the result of extension of *cotC* mRNA (see the text). The sequences shown in the vicinity of the 5′ terminus correspond to the non-transcribed DNA strand and the filled circles indicate the nucleotide corresponding to the 5′ terminus. (d) The time course and genetic dependence of the appearence of *cotC* mRNA. The primers Pr1 (lanes 1 to 6) and Pr2 (lanes 7 to 10) were used to generate extension products from total RNA from sporulating PY79 (spo^+) cells harvested at t_3 (lanes 4 and 8), t_8 (lanes 5 and 9), or t_{10} (lanes 6 and 10) or from KS450 (*gerE36*) cells harvested at t_3 (lane 1), t_8 (lane 2), or t_{10} (lanes 3 and 7).

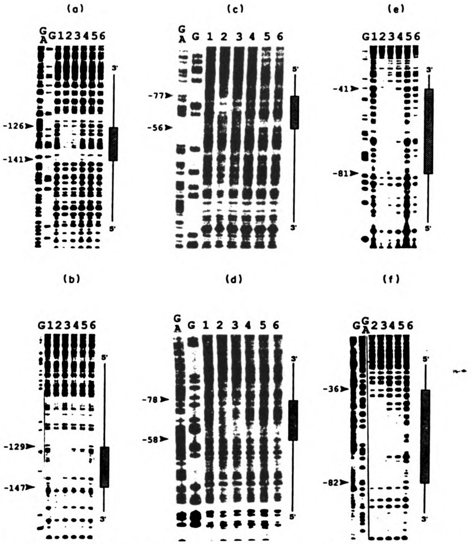

Figure 4. GerE footprints in *cotB* and *cotC* DNAs. Radioactive DNA fragments separately end-labeled on the transcribed or non-transcribed strand were incubated in separate reactions without protein (lane 6), with control protein (lane 1), or with 1 µg (lane 2), 0·5 µg (lane 3), 0·1 µg (lane 4), and 0·05 µg (lane 5) of GerE. After treatment with DNase I, the partially digested DNAs were separated by electrophoresis through an 8 M-urea/polyacrylamide gel alongside a sequencing ladder generated by chemical cleavage of the respective end-labeled DNAs. (a) and (b) Footprints of the non-transcribed (upper strand in Fig. 1) and the transcribed (lower) strands of *cotC*, respectively, using probes labeled at the *Hin*dIII site located upstream from the promoter. (c) and (d) Footprints of the non-transcribed (upper) and transcribed (lower) strands of *cotC*, respectively, using probes labeled at the *Ear*I site located downstream from the promoter. (e) and (f) Footprints of the non-transcribed (upper) and transcribed (lower) strands of *cotB*, respectively, using probes labeled at the *Eco*RI site located upstream from the promoter. The experiments of (a), (b), (e) and (f) were carried out using method (1) in Materials and Methods, and the experiments of (c) and (d) were carried out using method (2), although in other experiments (data not shown) similar footprinting results to those seen in (a) and (b) were obtained using method (2).

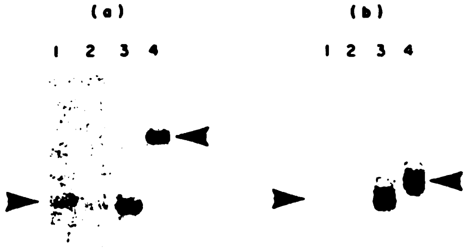

Figure 5. GerE stimulates *cotB* and *cotC* transcription *in vitro*. Template DNA (0·4 pmol) was transcribed with partially purified σ^K RNA polymerase (0·2 μg) alone, or with control protein or GerE (0.4 μg) added immediately after the addition of RNA polymerase. Run-off transcripts were electrophoresed in 5% polyacrylamide gels containing 8 M-urea and were detected by autoradiography. Arrowheads denote the positions of run-off transcripts of the expected sizes in each panel, as judged from the migration of end-labeled DNA fragments of *Msp*I-digested pBR322. (a) *cotB* transcription from replicative form DNA of a recombinant phage containing the *cotB* promoter region. The phage DNA was linearized with *Bam*HI (lanes 1 to 3, 177-base transcript) or *Hind*III (lane 4, 207-base transcript) and transcribed with σ^K RNA polymerase alone (lane 1), or with control protein (lane 2) or GerE (lanes 3 and 4) added. (b) *cotC* transcription from restriction fragments isolated from pHI1. The *Hind*III/*Xba*I fragment (lanes 1 to 3, 174-base transcript) or the *Hind*III/*Sal*I fragment (lane 4, 180-base transcript) were transcribed with σ^K RNA polymerase alone (lane 1), or with control protein (lane 2) or GerE (lanes 3 and 4) added.

tion fragment was used as the template for *in vitro* transcription, σ^K RNA polymerase produced run-off transcripts of the expected sizes from *cotC* in the presence of GerE (Fig. 5(b), lanes 3 and 4). A very weak signal was observed in the presence of control protein (lane 2) or with no addition (lane 1) in a longer autoradiographic exposure than that shown in Figure 5(b). Primer extension analysis of the GerE-stimulated *cotC* transcript produced by σ^K RNA polymerase *in vitro* demonstrated that it had the same 5′ terminus as *cotC* mRNA produced *in vivo* (Fig. 3(c)).

(e) *Effects of GerE on* in vitro *transcription of other mother-cell-expressed genes*

σ^K RNA polymerase partially purified from a *gerE* mutant has been shown to transcribe from the *cotD* and *sigK* (previously called *spoIVCB*) promoters *in vitro* (Kroos *et al.*, 1989). GerE stimulated *cotD* transcription two- to threefold (Fig. 6(a)) and completely inhibited *sigK* transcription (Fig. 6(b)) by partially purified σ^K RNA polymerase. Although the effect of GerE on *cotD* transcription was modest, a two- to threefold stimulation was consistently observed in four independent experiments (data not shown). The level of stimulation was not further enhanced by the use of twice as much GerE as that employed in the experiment of Figure 6(a) (data not shown).

The partially purified σ^K RNA polymerase produced run-off transcripts of the expected sizes from *gerE* (Fig. 6(c), lanes 1 and 2) and from *cotA* (Fig. 6(d), lanes 1 and 2). These transcripts were also produced by σ^K RNA polymerase reconstituted

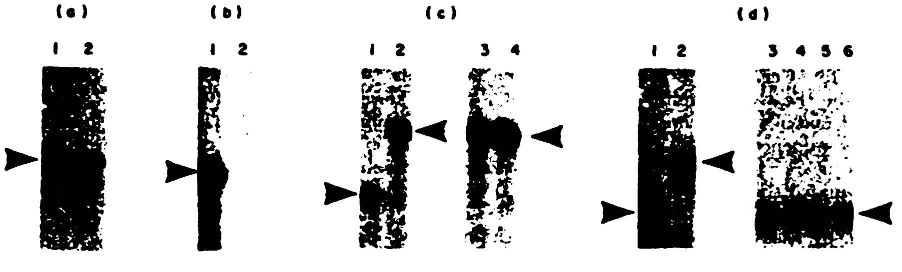

Figure 6. Effects of GerE on *cotD*, *sigK*, *gerE* and *cotA* transcription *in vitro*. Linearized plasmid DNA (2 μg) was transcribed with partially purified σ^K RNA polymerase (0·2 μg) alone, or with control protein or GerE (0·4 μg) added immediately after the addition of RNA polymerase. Run-off transcripts were electrophoresed in 5% polyacrylamide gels containing 8 M-urea and were detected by autoradiography. Arrowheads denote the positions of run-off transcripts of the expected sizes in each panel, as judged from the migration of end-labeled DNA fragments of *Msp*I-digested pBR322. (a) *cotD* transcription from *Hind*III-digested pLRK100 (225-base transcript) with σ^K RNA polymerase and control protein (lane 1) or GerE (lane 2). (b) *sigK* transcription from *Xba*I-digested pBK16 (170-base transcript) with control protein (lane 1) or GerE (lane 2). (c) *gerE* transcription from pSC146 digested with *Bam*HI (lane 1, 174-base transcript) or *Hind*III (lanes 2 to 4, 204-base transcript) with σ^K RNA polymerase alone (lanes 1 and 2), or with control protein (lane 3) or GerE (lane 4) added. (d) *cotA* transcription from pKS23 digested with *Nco*I (lane 1, 131-base transcript) or *Eco*RI (lane 2, 149-base transcript), from *Nco*I-digested pKS22 (lanes 3 and 4, 131-base transcript), and from *Nco*I-digested pKS24 (lanes 5 and 6, 131-base transcript) with σ^K RNA polymerase alone (lanes 1 and 2), or with control protein (lanes 3 and 5) or GerE (lanes 4 and 6) added. [α-^{32}P]UTP was the labeled nucleotide in the experiments shown in (c) and (d).

from gel-purified σ^K and *B. subtilis* core RNA polymerase (data not shown). GerE protein had no effect on *gerE* transcription *in vitro* by partially purified σ^K RNA polymerase (Fig. 6(c), lane 4). The effect of GerE on *cotA* transcription *in vitro* varied depending on the particular DNA template used (Fig. 6(d)). GerE had little effect on *cotA* transcription from a template containing 115 bp of DNA upstream from the *cotA* transcriptional startsite (lane 6); however, GerE inhibited *cotA* transcription approximately twofold (in 2 independent experiments) from a template containing approximately 430 bp of upstream DNA (lane 4).

Discussion

(a) *Transcriptional activation by GerE*

We have identified binding sites for GerE at the 5′ ends of *cotB* and *cotC*, coat protein genes whose transcription depends on the appearance of GerE during sporulation. We have also shown that GerE stimulates *cotB* and *cotC* transcription *in vitro* by σ^K RNA polymerase and that the *gerE* gene itself can be transcribed *in vitro* by σ^K RNA polymerase. These results support the view (Zheng & Losick, 1990) that in the mother cell, σ^K RNA polymerase first directs the transcription of *gerE*, then acts in conjunction with the product of *gerE* to direct the transcription of *cotB*, *cotC* and, perhaps, other late-activated sporulation genes.

Interestingly, the region of GerE-conferred protection from DNase I action in *cotB* (41 to 47 bp) was approximately twice the length of the two separate protected regions in *cotC* (16 to 19 bp for binding site 1 and 21 to 22 bp for binding site 2). Our interpretation of this observation is that *cotB* contains tandem GerE binding sites and that *cotC* contains two separate GerE binding sites. Inspection of the sequences in the protected regions reveals three similar 5 bp sequences, two (TGGGT and TAGGC) in *cotB* and one (TGGGC, found in binding site 1) in *cotC*. If these are recognition sequences for GerE, then a possible consensus sequence for the GerE binding site is TPuGGPy. The closest match to this consensus in *cotC* binding site 2 is the sequence TGGAC. Nevertheless, binding site 2 appears to be sufficient to mediate GerE-stimulated transcription of *cotC*, since a DNA template with less than half of binding site 1 (produced by cleavage with *Hae*III at position −133) retains the ability to be transcribed *in vitro* by σ^K RNA polymerase in the presence of GerE (R.H. & L.K., unpublished results).

The downstream boundaries of the GerE binding sites in *cotB* (position −36) and in *cotC* (position −56 for binding site 2) are immediately adjacent to or near regions of DNA that are expected to interact with RNA polymerase (that is, the promoters). By analogy with the positioning of the binding sites for several well-characterized, positively acting regulatory proteins, the DNA-bound GerE can be considered to be appropriately positioned to stimulate RNA polymerase to transcribe from the *cotB* and *cotC* promoters. For example, the binding region (the tandem operator sites O_{R2} and O_{R1}) for the phage λ repressor is located between 34 and 74 bp upstream from the transcriptional startsite for the promoter (P_{RM}) from which the repressor stimulates transcription of its own structural gene (*cI*) by *E. coli* RNA polymerase (Johnson *et al.*, 1979; Meyer & Ptashne, 1980). As another example, the catabolite gene activator protein (CAP) binds to sequences (typically protecting about 25 bp) centered from about 41 to 107 bp upstream from the transcriptional startsite of genes whose transcription it stimulates (de Crombrugghe *et al.*, 1984).

An added significance of our demonstration *in vitro* that GerE can bind to and stimulate transcription from the regulatory regions of genes under its control is that GerE is the prototypical representative of the putative regulatory domain of a large and diverse group of procaryotic transcriptional activator proteins. Thus, GerE exhibits high overall similarity to the COOH-terminal regions of certain regulator members (the BvgA sub-group, which includes DegU, ComA and FixJ) of the family of two-component sensor and response regulator systems in bacteria (Gross *et al.*, 1989; Kahn & Ditta, 1991). The NH_2-terminal region of the response regulator proteins contains a site for phosphorylation by the sensor component of the two-component system, and its phosphorylation state influences the activity of the COOH-terminal domain with respect to transcriptional activation (Kofoid & Parkinson, 1988; Nixon *et al.*, 1986). Strikingly, GerE, which lacks the NH_2-terminal domain, is highly similar along its entire 72 amino acid length to the COOH-terminal domain of the BvgA sub-group of response regulator proteins. No member of the BvgA sub-group has (to our knowledge) been shown to bind to DNA or activate transcription *in vitro*, but our results reinforce the view that the GerE-like, COOH-terminal domain of these proteins is directly responsible for the transcriptional activation of genes under the control of this sub-group of response regulator proteins. Likewise, the similarity of GerE to the COOH terminus of *E. coli* MalT, a large regulatory protein whose NH_2-terminal region is dissimilar to the phosphorylation domain of the two-component regulator proteins (Gross *et al.*, 1989), once again suggests that the GerE-like region of MalT could be responsible for DNA-binding and transcriptional activation by this regulatory protein (Richet *et al.*, 1991; Vidal-Ingigliardi *et al.*, 1991). Finally, the similarity of GerE to the COOH-terminal domain (region 4) of sigma factors (Kahn & Ditta, 1991) reinforces the view that this domain mediates the recognition of the −35 region of promoters (Gardella *et al.*, 1989; Siegele *et al.*, 1989).

(b) *Consensus sequence for promoters recognized by σ^K RNA polymerase*

σ^K RNA polymerase has been shown to transcribe from the *cotD* and *sigK* (previously called *spoIVCB*)

```
                 -35                    -10
Consensus        AC     - 17 bp -   CATA---TA

sigK      cggtacagacACagacagcctcccggtcaCATAcatTtacatataggc
cotA      atttttgtaACcatcacgtccttattgtCATtaacTAtagtaccaat
cotD      ttgcatcagaACatgtacccccttatttttCATAactTAgtattgtaat
gerE      tgtaaacgtcACctcctgcgcccttcttaCATAtgaTAtctcgactat

cotB      ttgaattagttCaacaaataaatgtgacaCgTAtatatgcagtatgt
cotC      aactgtccaagCcgcaaaatc tactcgcCgTAtaaTAaagcgtagta
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Figure 7. Alignment of promoters transcribed by σ^K RNA polymerase. The nucleotide sequences of the *sigK*, *cotA*, *cotD* and *gerE* promoter regions (see the text for references) are aligned with respect to conserved nucleotides (capital letters) in the −10 and −35 regions relative to the transcriptional startsites (underlined). Shown above are the consensus −10 and −35 sequences, separated by 17 bp, and shown below are the *cotB* and *cotC* promoter regions with matches to the consensus indicated by capital letters (a 1 bp gap was introduced into the *cotC* sequence between the −10 and −35 regions).

promoters *in vitro* (Kroos *et al.*, 1989). Efficient transcription of *sigK* by σ^K RNA polymerase also required a small, DNA-binding protein that is the product of the *spoIIID* gene (Kroos *et al.*, 1989; Kunkel *et al.*, 1989). Using σ^K RNA polymerase reconstituted from gel-purified σ^K and *B. subtilis* core RNA polymerase, we find that *gerE* and *cotA*, like *cotD*, are transcribed efficiently by σ^K RNA polymerase in the absence of SpoIIID, findings that confirm and extend the results of studies on the regulation of these genes *in vivo* (Cutting *et al.*, 1989; Sandman *et al.*, 1988). As shown in Figure 7 and as noted previously (Foulger & Errington, 1991; Zheng & Losick, 1990), the promoter regions of *cotD* (Zheng & Losick, 1990), *gerE* (Cutting *et al.*, 1989), *cotA* (Sandman *et al.*, 1988) and *sigK* (Kunkel *et al.*, 1988) each contain sequences similar to CATA---TA at about position −10 relative to their transcriptional startsites. Figure 7 also shows that the *cotB* and *cotC* promoters, which were transcribed weakly by σ^K RNA polymerase in the absence of GerE, display some similarity to the CATA---TA sequence. Interestingly, the putative −10 consensus sequence for σ^K-recognized promoters is similar to the sequence CATACA-T, which is conserved in the −10 region of promoters transcribed by RNA polymerase containing the related sporulation sigma factor, σ^E (see Roels *et al.* (1992) and Foulger & Errington (1991) for recent compilations of promoters recognized by σ^E RNA polymerase). Unlike promoters recognized by σ^E RNA polymerase, however, promoters recognized by σ^K RNA polymerase that have been characterized to date display only a limited region of similarity to each other in their −35 regions. The sequence AC is, however, found 17 bp upstream from the −10 region in the four promoters transcribed by σ^K RNA polymerase in the absence of GerE, but only the C is found at the corresponding position in *cotB* and *cotC*. We note that three other promoters that are inferred to be transcribed by σ^K RNA polymerase in the absence of GerE, namely, *spoVJ*P2 (Foulger & Errington, 1991), *cotE*P2 (Zheng & Losick, 1990), and the promoter for the newly discovered coat protein gene *cotF* (Cutting *et al.*, 1991*b*), contain −35 and −10 sequences that strongly conform to the sequences AC and CATA---TA, respectively, at a spacing of 16 to 17 bp. Mutational analyses will be needed to determine whether the AC and CATA---TA sequences are important for promoter recognition by σ^K RNA polymerase.

(c) *Effect of GerE on the transcription of* cotD *and* sigK

GerE stimulated *cotD* transcription *in vitro* by σ^K RNA polymerase two- to threefold (Fig. 6(a)). This result is in qualitative agreement with the finding that *cotD-lacZ* expression is reduced about sevenfold in *gerE* mutant cells (Zheng & Losick, 1990). Inspection of the *cotD* promoter region (Zheng & Losick, 1990) reveals a 12 bp sequence (AAAATAGGTCTT) at positions −43 to −54 with ten matches to a sequence (positions −69 to −80) protected by GerE in the *cotB* promoter region (Fig. 1). Within the 12 bp sequence in the *cotD* promoter region is a 5 bp sequence (TAGGT) that conforms to the putative consensus binding sequence for GerE described above. It will be interesting to determine whether GerE binds to this sequence, since it would appear to position GerE appropriately to stimulate RNA polymerase, as discussed above.

GerE completely inhibited *sigK* transcription by partially purified σ^K RNA polymerase (Fig. 6(b)). The partially purified σ^K RNA polymerase used in this experiment contained a small amount of SpoIIID, thus permitting adequate transcription of *sigK*. Inhibition of *sigK* transcription by GerE was unexpected, since expression of a *sigK-lacZ* fusion was about normal in *gerE* mutant cells (Kunkel *et al.*, 1988). Inspection of the *sigK* promoter region (Kunkel *et al.*, 1988) reveals a 15 bp sequence (ACATATAGGCTTTTG) at positions −4 to +11 with 12 matches to a sequence (positions −41 to −55) protected by GerE in the *cotB* promoter region (Fig. 1). Within the 15 bp sequence in the *sigK* promoter region is a 5 bp sequence (TAGGC) that conforms to the putative consensus GerE binding sequence. In addition, this 5 bp sequence is repeated in inverted orientation at positions +11 to +15. Thus, there may be two GerE binding sequences near the start-site of *sigK* transcription and GerE bound at these sites may prevent σ^K RNA polymerase from transcribing *sigK*. If these sites do mediate repression of *sigK* transcription by GerE, it could explain why a *sigK-lacZ* fusion was expressed equally in wild-type or *gerE* mutant cells, since the fusion was created by insertion of a transposon (Tn*917lac*) 4 bp downstream from the *sigK* tran-

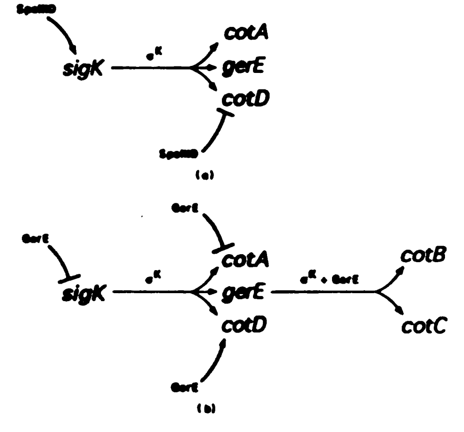

Figure 8. Regulatory effects of SpoIIID and GerE during stages IV and V of sporulation. The effects of SpoIIID and GerE on transcription by σ^K RNA polymerase *in vitro* are illustrated. As noted in the text, studies *in vivo* also support some of the regulatory effects depicted. (a) During stage IV of sporulation, SpoIIID stimulates transcription of *sigK* and inhibits transcription of *cotD*. The *gerE* gene is transcribed by σ^K RNA polymerase. (b) During stage V of sporulation, GerE inhibits transcription of *sigK* and *cotA*, and stimulates transcription of *cotD*, *cotB* and *cotC*.

scriptional startsite (Kunkel *et al.*, 1988), and this would have presumably disrupted the putative GerE binding site.

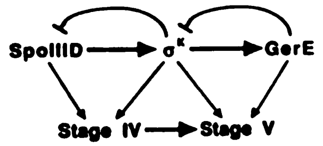

Figure 9. Regulatory interactions controlling the levels of SpoIIID, σ^K and GerE govern the stage IV to V transition in the mother cell. SpoIIID stimulates *sigK* transcription, leading to σ^K production (Kroos *et al.*, 1989). σ^K RNA polymerase transcribes *gerE*, leading to GerE production. The appearance of σ^K causes a decrease in the level of SpoIIID (Halberg & Kroos, 1992). GerE inhibits transcription of *sigK*, down-regulating σ^K production. Thus, σ^K RNA polymerase functions during both stage IV and stage V, but a declining level of SpoIIID and a rising level of GerE switch the pattern of σ^K-directed gene expression from the stage IV pattern to the stage V pattern.

(d) *Opposite effects of GerE and SpoIIID help to drive the mother-cell regulatory cascade*

GerE and SpoIIID exert opposite effects on σ^K-directed transcription of both *cotD* and *sigK* (Fig. 8). It has been shown that SpoIIID stimulates *sigK* transcription and inhibits *cotD* transcription *in vitro* (Kroos *et al.*, 1989; and Fig. 8(a)). These properties of SpoIIID led to the suggestion that inactivation or sequestering of SpoIIID during sporulation causes a switch from transcription of *sigK* (and perhaps other stage IV sporulation genes) to transcription of *cotD* (and perhaps other stage V genes) (Kroos *et al.*, 1989). Recently, it has been shown that the level of SpoIIID decreases at the appropriate time during sporulation to cause such a switch (Halberg & Kroos, 1992). Furthermore, the decrease in the level of SpoIIID correlates with the appearance of σ^K, suggesting that the appearance of σ^K initiates the switch. We have shown here that σ^K RNA polymerase transcribes *gerE* (Fig. 6(c)). Thus, the appearance of σ^K RNA polymerase beginning at about hour 4 of sporulation (Cutting *et al.*, 1989; Lu *et al.*, 1990) would result not only in a declining level of SpoIIID, but also in a rising level of GerE. We have also shown here that GerE inhibits *sigK* transcription (Fig. 6(b)) and stimulates *cotD* transcription (Fig. 6(a)) by σ^K RNA polymerase *in vitro* (Fig. 8(b)). Because GerE exerts the opposite effects of SpoIIID on σ^K-directed transcription of *sigK* and *cotD*, the appearance of GerE would reinforce the switch in the pattern of mother-cell gene expression previously postulated to be brought about by inactivation or sequestering of SpoIIID. The reason for the apparent redundancy in the switch is unclear. Perhaps SpoIIID prevents premature expression of *cotD* (and perhaps other stage V genes) during the stage (IV) of spore cortex formation so that the σ^K produced initially directs expression of *sigK* (autogenous regulation), *gerE*, and genes involved in cortex formation (Halberg & Kroos, 1992). Accumulation of GerE would terminate this period by diverting σ^K RNA polymerase away from transcription of *sigK* (and perhaps other stage IV genes) and would initiate the stage (V) of spore coat formation by directing σ^K RNA polymerase to transcribe genes encoding spore coat proteins. According to this model, the regulatory interactions illustrated in Figure 9 co-ordinate the levels of SpoIIID, σ^K and GerE so as to produce a molecular switch governing the transition from the stage IV pattern of mother-cell gene expression to the stage V pattern.

The effects of GerE on transcription of *gerE* and *cotA* by σ^K RNA polymerase *in vitro* are consistent with the effects of a *gerE* mutation on expression of these genes *in vivo*. GerE had no effect on transcription of the *gerE* gene *in vitro* (Fig. 6(c)) and expression of a *gerE-lacZ* fusion is normal in a *gerE* mutant (Cutting *et al.*, 1989). The effect of GerE on *cotA* transcription *in vitro* varied from little effect with a template containing 115 bp of DNA upstream from the transcriptional startsite to a modest (but reproducible), twofold inhibition with a template containing approximately 430 bp of upstream DNA (Fig. 6(d)). If GerE inhibits *cotA* transcription by binding to DNA, this result suggests that it must do so by binding to a site(s) more than 115 bp upstream from the startsite of transcription. In a previous study (Cutting *et al.*

1989), a *cotA-lacZ* fusion containing as little as 300 bp of DNA upstream from the *cotA* transcriptional startsite was found to be expressed about threefold higher in a *gerE* mutant relative to wild-type cells.

In summary, we have presented biochemical evidence that GerE is a regulatory protein capable of either stimulating or inhibiting transcription of particular genes in the mother-cell line of gene expression. In this respect, GerE appears to be analogous to SpoIIID; however, the ordered appearance of first SpoIIID, then GerE, and the opposite effects of these two proteins on the transcription of genes like *sigK* and *cotD* presumably ensures proper flow of the regulatory cascade (Zheng & Losick, 1990) controlling mother-cell gene expression. In the cases of *cotB* and *cotC*, GerE binds to specific sequences immediately adjacent to the promoter and stimulates transcription by σ^K RNA polymerase. This finding supports the previous proposal (Zheng & Losick, 1990) that GerE directly activates the expression of genes in the terminal temporal class of mother-cell expressed genes.

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