The Mechanism of Transcription Activation by the *Bacillus subtilis* Response Regulator, Spo0A.

by

Dean Allistair Rowe-Magnus

B.Sc., University of Ottawa, 1991
M.Sc. University of Toronto, 1994

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

Department of Microbiology and Immunology

We accept this thesis as conforming to the required standard

UNIVERSITY OF BRITISH COLUMBIA

November, 1998

© Dean Allistair Rowe-Magnus, 1998
In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of **Microbiology**

The University of British Columbia
Vancouver, Canada

Date 1/2/98

DE-6 (2/88)
ABSTRACT.

In Bacillus subtilis, signals which indicate nutrient depletion initiate a complex series of events leading to endospore formation. Environmental signals are detected by multiple sensor kinases and a cellular response is elicited by phosphorylation of the transcription regulator, Spo0A, which initiates endospore formation by specifically stimulating or repressing expression of developmental genes. In this thesis, the in vitro properties of Spo0A have been examined using the promoter for the spollG operon as a target. In vivo expression from the spollG promoter is absolutely dependent on phosphorylated Spo0A and RNA polymerase containing the major sigma subunit, σA.

As a first step, a comparison of the activation characteristics of phosphorylated Spo0A (Spo0A-P) and a constitutively active form of Spo0A consisting of the C-terminal 124 amino acids (Spo0ABD) was carried out. Both Spo0A-P and Spo0ABD stimulated transcription from the spollG promoter 10 fold more efficiently than Spo0A. DNAse I footprint assays revealed that phosphorylation enhanced binding of intact Spo0A to the 0A boxes, while the binding of Spo0ABD was similar to that of Spo0A. Thus, the activation of Spo0A is not primarily due to enhanced DNA binding. Experiments testing the effects of ionic strength indicated that the presence of a phosphorylated N-terminus increased the stability of Spo0A-RNA polymerase complexes at the spollG promoter.

Potassium permanganate probing revealed that when activated Spo0A was incubated with RNA polymerase and a DNA fragment containing the spollG promoter, base pairs between -13 and -3, relative to the start site of transcription, were denatured. Bases downstream of -3 were exposed in the presence of initiating nucleotides. Addition of activated Spo0A or RNA
polymerase alone did not induce denaturation. Heteroduplex templates that contained the non-template sequence of the wild type promoter on both strands between positions -3 and -13 were efficiently transcribed without activated Spo0A. These data suggested that DNA strand separation was a two step process and that the activation of Spo0A created a form that interacted with the polymerase to induce the first of the two steps.

In vitro transcription from the spollG promoter by Bacillus subtilis RNA polymerase reconstituted with wild type alpha subunits or with C-terminal deletion mutants of alpha was examined. Wild type or mutant polymerases were stimulated by activated Spo0A although some differences in the interaction of the enzymes with the DNA were noted.

Mutational analysis of the C-terminus of Spo0A identified 3 residues required for activation of the spollG promoter. These and other data support a proposal in which activation of Spo0A triggers a structural change in the regulator that exposes a RNA polymerase interaction domain. Contacts between activated Spo0A and RNA polymerase induce the upstream denaturation event and it is proposed that stimulation of the spollG promoter by Spo0A occurs by accelerating this denaturation step.
# TABLE OF CONTENTS

**ABSTRACT.**

**LIST OF TABLES.**

**LIST OF FIGURES.**

**ABBREVIATIONS AND SYMBOLS.**

**ACKNOWLEDGMENTS.**

**INTRODUCTION.**

1.0. The dynamics of transcription.  
1.1. A general pathway for transcription initiation.  
1.2. Promoter elements.  
1.3. RNA polymerase.  
1.3.1. The α subunit.  
1.3.2. The σ subunit  

2.0. Sporulation in *Bacillus subtilis*.  
2.1. Induction of sporulation.  
2.2. Two-Component regulators.  
2.3. The phosphorelay.  
2.4. Promoters regulated by Spo0A.  
2.4.1. Phosphorelay components.  
2.4.2. The sigma factor cascade.  
2.4.3. The *spollG* promoter.  

3.0. A model for Spo0A activation of the *spollG* promoter.  
3.1. Mechanisms of activation by positive regulators.  
3.1.2. Lambda repressor (λcl).  
3.1.3. PhoB.  
3.1.4. Nitrogen regulatory protein C (NtrC).
3.2. The characteristics of Spo0A. 30
4.0. Experimental rationale. 32

EXPERIMENTAL PROCEDURES. 34
1.0. Bacterial strains and media. 34
2.1. Wild type spollG template. 34
2.2. RNA polymerase purification. 34
2.3. Phosphorylation of Spo0A. 35
2.4. In vitro transcription assays. 36
3.0. Primer extension analysis. 37
4.0. KMnO₄ footprint reactions. 38
5.0. DNase I footprint reactions. 39
6.0 Electrophoretic mobility shift assays. 40
7.0. Mutation of the spollG promoter. 40
7.1. Construction of heteroduplex templates. 41
8.0. Mutation of the spoOA gene. 49
8.1. Screening for sporulation defective mutants of Spo0A. 49
8.2. Determination of the sporulation frequency. 53
8.3. β-galactosidase assays. 53

RESULTS 55
1.0. Characterizing Spo0ABD. 55
1.1. Heparin-resistant complex formation at spollG. 55
1.2. Comparison of the stimulatory properties of Spo0A, Spo0A~P and Spo0ABD. 59
1.2.1. Stimulation of heparin-resistant complex formation measured by transcript production in single round assays. 59
1.2.2. Stimulation of heparin-resistant complex formation measured by electrophoretic mobility shift assay (EMSA). 60

1.2.3. Stimulation of the rates of transcription. 61

1.2.4. Temperature dependence of spollG transcription initiation. 68

1.2.5. Effect of anions on transcription stimulation. 71

1.2.6. DNA binding by Spo0A, Spo0A–P and Spo0ABD. 73

2.0. The mechanism of transcription stimulation by activated Spo0A. 79

2.1. Structural probing of open complex formation with potassium permanganate (KMnO₄). 79

2.1.1. The pathway of promoter melting at spollG. 89

2.2. Transcription by RNA polymerase from heteroduplex templates. 91

2.2.1. Temperature dependence of transcription from heteroduplex templates. 95

3.0. Interaction of Spo0A with the C-terminus of the alpha subunit of RNA polymerase. 101

3.1. Effect of deletion mutants of the alpha subunit on the mechanism of initiation. 103

3.2. EMSA detection of open complex formation by RNA polymerase containing deletion mutants of the α subunit. 108

4.0. Positive control mutations in the C-terminus of Spo0A. 111

DISCUSSION. 119

1.0. The pathway of open complex formation at spollG. 120

1.1. Establishment of the denatured region upstream of the transcription start site. 121

1.2. The initiation of RNA synthesis. 122

1.3. Start site selection in the initiation of transcription. 124

1.4. Open complex formation follows a melting-from-the-edge pattern. 125

2.0. How Spo0A–P might stimulate transcription. 127
2.1. Repositioning the σ subunit. 127
2.2. A conformational change in the RNA polymerase σ subunit. 128
3.0. The Spo0A~P paradigm. 130
3.1. Comparison of activation by Spo0A to activation by other transcription activators. 133
3.2. Transcription from non-consensus promoters. 134
4.0. The role of the α subunit of RNA polymerase in transcription. 135
5.0. Activating regions in σA, σH and the C-terminus of Spo0A. 139
5.1. A region in Spo0A required for PspollG stimulation. 140
5.2. The possible effects of the region 1 and 2 mutations in Spo0A on σA-dependent transcription. 142
5.3. The potential effect of the region 1 and 2 mutations in Spo0A on σH-dependent transcription. 143
6.0. Potential consequences of phosphorylation on Spo0A. 143
6.1. Effects of phosphorylation on DNA binding. 144
6.2. The hydrophobic effect. 145
6.3. Dimerization of Spo0A~P. 149
6.4. A proposal for Spo0A~P interaction with RNA polymerase. 150
7.0. Negative regulators of initiation. 150
7.1. Spo0JA. 151
7.2. SinR. 152
8.0. Unanswered questions. 152
8.1. Reconciling the effects of phosphorylation on stimulation of σA and σH-dependent transcription. 152
8.2. N-terminal inhibition of the output domain. 153
8.3. Identification of the DNA binding region of Spo0A. 154
8.4. Regulation of spollG transcription by the promoter structure. 154
8.5. DNA untwisting. 155
8.6. Open complex formation at spollG as a tool to study the beginnings of DNA strand separation.

REFERENCES
**LIST OF TABLES**

Table 1. Mutagenic Primers used in mutational PCR of the *spollG* promoter and *spo0A*.

Table 2. Plasmids produced and used in this study.

Table 3. Bacterial strains created and used in this study.

Table 4. DNA templates used in transcription studies.

Table 5. Amino acid sequences of the C-terminal mutants of Spo0A.

Table 6. The effects of Spo0A mutations on spore formation.
LIST OF FIGURES

Figure 1. Basic features of the transcription unit and the interaction of RNA polymerase with the promoter. 4

Figure 2. Alignment of the C-terminal amino acids of the wild type *E. coli* and *B. subtilis* α subunits of RNA polymerase. 7

Figure 3. *E. coli* activator contact regions on RNA polymerase and promoters. 10

Figure 4. The sporulation decision of *Bacillus subtilis*. 12

Figure 5. Two-component signal transduction systems. 14

Figure 6. Transcription interaction among genes of the phosphorelay and the initiation of the sigma factor cascade. 18

Figure 7. The sigma factor cascade and compartmentalized gene expression. 22

Figure 8. Features of the *spollG* promoter. 26

Figure 9. Map of the activating regions of Spo0A. 31

Figure 10. Creation of the heteroduplex templates. 45

Figure 11. Structure of the heteroduplex templates. 48

Figure 12. Mutation and cloning of the *spo0A* gene. 50

Figure 13. Transformation of mutant *spo0A* alleles into *B. subtilis*. 51

Figure 14. Heparin-resistant complex formation at the *spollG* promoter. 57

Figure 15. *In vitro* stimulation of transcription by Spo0A–P and Spo0ABD from *PspollG*. 62

Figure 16. EMSA assay of Spo0A, Spo0A–P and Spo0ABD stimulation of heparin-resistant complex formation. 63

Figure 17. EMSA assay of uninitiated complex formation Spo0A, Spo0A–P and Spo0ABD. 65

Figure 18. Kinetics of heparin-resistant complex formation. 69

Figure 19. Temperature-dependence of transcription from *PspollG*. 70
Figure 20. Influence of anion type and concentration on transcription from *PspollG*. 74

Figure 21. A phosphorylated N-terminus contributes to stable ternary complex formation. 75

Figure 22. Salt-stable complex formation by Spo0A-P. 76

Figure 23. Activator binding at the *spollG* promoter. 80

Figure 24. DNA strand separation at the *spollG* promoter. 85

Figure 25. KMnO₄ probing of the top (non-template) strand at the *spollG* promoter during the initiation of transcription. 87

Figure 26. Effect of temperature on DNA denaturation induced by Spo0ABD. 90

Figure 27. Mapping of the transcriptional start site from heteroduplex templates. 96

Figure 28. Transcription products from heteroduplex templates. 98

Figure 29. Temperature-dependence of transcription from heteroduplex templates. 100

Figure 30. Spo0A-P stimulation of transcription by RNA polymerase containing wild type or mutant alpha subunits. 102

Figure 31. Denaturation of the *spollG* promoter region induced by Spo0ABD plus RNA polymerase containing wild type or mutant alpha subunits. 105

Figure 32. Electrophoretic mobility shift assays of complexes formed by RNA polymerase containing mutant and wild type alpha subunits. 109

Figure 33. The effects of *spoOA* mutations on expression of a *spollG-lacZ* transcriptional fusion. 115

Figure 34. The model for Spo0A-P stimulation of the *spollG* promoter. 131

Figure 35. Amino acid sequence of the C-terminal domain of Spo0A. 137

Figure 36. Comparison of the N-terminal sequences of Spo0A and CheY. 147
# ABBREVIATIONS AND SYMBOLS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac^-</td>
<td>acetate anion</td>
</tr>
<tr>
<td>αCTD</td>
<td>carboxy-terminal domain of the alpha subunit (α) of RNA polymerase</td>
</tr>
<tr>
<td>αNTD</td>
<td>amino-terminal domain of the alpha subunit (α) of RNA polymerase</td>
</tr>
<tr>
<td>C1</td>
<td>RNA polymerase-( spollG ) promoter complex</td>
</tr>
<tr>
<td>CII</td>
<td>RNA polymerase-( spollG )-SpoOA promoter complex</td>
</tr>
<tr>
<td>CIII</td>
<td>RNA polymerase-( spollG )-Spo0A-P promoter complex</td>
</tr>
<tr>
<td>Cl^-</td>
<td>chloride anion</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>HR</td>
<td>heparin-resistant</td>
</tr>
<tr>
<td>KAc</td>
<td>potassium acetate</td>
</tr>
<tr>
<td>K_B</td>
<td>DNA binding constant</td>
</tr>
<tr>
<td>k_f</td>
<td>forward isomerization rate constant</td>
</tr>
<tr>
<td>KGlut</td>
<td>potassium glutamate</td>
</tr>
<tr>
<td>NTPs</td>
<td>nucleoside triphosphates</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxynucleoside triphosphates</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>( PsollG )</td>
<td>promoter for the ( spollG ) operon of ( Bacillus subtilis )</td>
</tr>
<tr>
<td>Spo0ABD</td>
<td>C-terminal fragment of ( Spo0A )</td>
</tr>
<tr>
<td>Spo0A-P</td>
<td>phosphorylated form of ( Spo0A )</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENTS.

Well! What a tour of duty! First off, I am indebted to my commander and chief, the big Kahuna, Dr. G. B. Spiegelman. Thanks for giving me a chance. Your imparted wisdom will not go to waste, unless the price of single malt scotch plummets. I would like to thank Dr. T. Warren for his critical (and I mean critical) reading of my thesis and his, what's the right word...ah - enigmatic sense of humor. Keep flashing those knees Dr. W. Of course, I would also like to thank the remaining members of my committee, Dr. T. Beatty and Dr. B. Finlay for their guidance and support. I also owe a great deal of thanks to the labs of Dr. J. Hoch and Dr. M. Salas for providing me the with purified phosphorelay components and the mutant RNA polymerase holoenzymes, respectively.

Send in the clowns! Vincent "J. J." Martin, we had some good times, eh? Next summer, we'll teach those Frenchmen how to party. Martina "margarita" Ochs-whatever-the-rest-of-it-is, thanks for lending me your ear, offering your advice and being the awesome friend that you are. I'll miss you both. Maggie, keep dancing on the minefield, girl and don't you dare come and visit me. Loverne, supertech, superwoman, superb. I'll never forget you (I'll let you know if a hotdog is male or female en français) or that wonderful daughter of yours. Gillian, I don't remember what it was you whipped up for dinner, but it was some kick-butt sustenance. Do you deliver? Oh by the way, do frozen agarose gels run better? And to the most eclectic cast and crew of professors and office personnel ever to be assembled into a functional (well, close enough) unit, thanks so very much.

And to my family; my mom and my brothers, Patrick and Basil. I could not have done it without you. This thesis is for you (cause no one else seems to want it).
INTRODUCTION.

1.0. The dynamics of transcription.

Information stored in DNA directs the synthesis of proteins. The process of using the information begins with transcription. The control of information flow at the level of transcription is a major mechanism for controlling development in prokaryotes and eukaryotes. In this thesis, one example of transcription control is explored. The model system used is transcription stimulation from the spolIG promoter of Bacillus subtilis. Transcription of the spolIG promoter requires the activated form of a specific regulator, Spo0A, and RNA polymerase containing the major sigma subunit, σ^A. The introduction reviews transcription initiation, sporulation and regulation control. A brief review of the factors governing gene expression is presented below.

1.1. A general pathway for transcription initiation.

Transcription initiation in bacteria has been most studied for E. coli RNA polymerase (Buc and McClure, 1985; Roe et al., 1985; Leirmo and Record, 1990). A general pathway for transcription initiation has been proposed and the basic steps that lead to transcription by RNA polymerase are described below (Leirmo and Record, 1990; deHaseth et al., 1998).

\[
\begin{align*}
K_B & \quad k_{1} & \quad k_{2} & \quad \text{NTPs} \\
R + P & \leftrightarrow RP_C & \leftrightarrow RP_n & \leftrightarrow RP_0 & \leftrightarrow RP_{ITC} & \leftrightarrow RP_{IEC} & \rightarrow RNA \\
(2-10 \text{ nts})
\end{align*} \tag{1}
\]

In the initiation reaction, RNA polymerase (R) binds to the promoter (P) to form a competitor-sensitive closed complex (RP_C). The affinity of RNA polymerase for a promoter is described by the binding constant, \( K_B \).
RNA synthesis uses only one of the two DNA strands as a template to which incoming NTPs base pair. However, this process is blocked by the hydrogen bonds between the DNA base pairs of the double helix. To proceed, disruption of the bonds between the DNA strands to form the transcription bubble must occur in a process termed melting (for review see deHaseth and Helmann, 1995). The ease with which the transcription bubble is established is dependent upon several factors, including the DNA sequence and structure, the extent of supercoiling, and the influence of accessory proteins. Transition from a closed to a stable open complex (RP₀), in which the DNA around the transcription start site is denatured, may include several intermediates (RPₙ) containing distorted or partially melted DNA (for review see deHaseth and Helmann, 1995). Rate constants for these transitions are indicated as $k_{fin}$.

NTPs favor the downstream propagation of the denatured region and stimulate the RPₙ to RP₀ transition. The stability of RP₀ is promoter-dependent and RNA polymerase may not form stable complexes unless one to several phosphodiester bonds are formed (Dobinson and Spiegelman, 1987; Rojo et al., 1993; Chen and Helmann, 1997; Bird et al., 1993; Ohlsen and Gralla, 1992; Whipple and Sonenshein, 1992; Wang and Gralla, 1996). RNA polymerization then proceeds in the presence of NTPs to generate a complex, RP_{ITC}, (initial transcribing complex) containing RNA transcripts of 2-10 nucleotides in length. Under appropriate conditions, promoter escape by RNA polymerase converts these ITCs to initial elongating complexes (RP_{IEC}).

Some transcription activators, for example CAP at the lac promoter, increase promoter activity by increasing the $K_B$ (Malan et al., 1984). Others, like λcl, act by enhancing the rate of isomerization from a closed to an open complex, $k_f$, at one or more intermediates (Li et al., 1997). Bifunctional activators, such as CAP at the gal promoter (Bell et al., 1990b) and λcll (Ho et
al., 1982; Shih and Gussin, 1983) affect both $K_B$ and $k_f$. The mechanism employed by the activator to stimulate promoter activity may involve conformational changes in the DNA (Ansari et al., 1992), RNA polymerase (Wang et al., 1995; Wedel and Kustu, 1995) or both.

1.2. Promoter elements.

The transcription process begins with the binding of RNA polymerase to the promoter to form the closed complex. Specific promoter recognition depends on a protein subunit of RNA polymerase subunit called sigma ($\sigma$; see below). I will focus the introduction on the major vegetative sigma factors, $\sigma^{70}$ and $\sigma^A$ of *E. coli* and *B. subtilis*, respectively, since the work in the thesis concerns $\sigma^A$ of *Bacillus subtilis*. The two hexamer sequences recognized by RNA polymerase containing $\sigma^{70}/\sigma^A$ are the -10 and -35 basal elements, so called because of their location relative to the start site of transcription, +1 (Figure 1). The -35 site is proposed to be necessary for the initial binding of RNA polymerase to the promoter and the -10 site is where nucleation of DNA strand separation occurs (for review see deHaseth and Helmann, 1995; deHaseth et al., 1998). The length of the DNA separating the -10 and -35 elements, designated the spacer DNA, can drastically affect promoter activity. A spacer length of 17-18 bp is optimal for promoter activity. Optimal spacer length has been proposed to reflect interaction of regions 2.4 and 4.2 of $\sigma$ (Dombroski et al., 1996; see below). Single base pair insertions or deletions that change the optimal spacing are deleterious *in vitro* and *in vivo* (Stefano and Gralla, 1982; Mulligan et al., 1985; Ayers et al., 1989).

Recently, two other influential characteristics of promoter structure have been described. One of these determinants is an AT rich region positioned between -40 an -60 of some promoters termed the upstream (UP) element. This
Figure 1. Basic features of the transcription unit and the interaction of RNA polymerase with the promoter. The subunits of RNA polymerase holoenzyme ($\beta$, $\beta'$, $\alpha$ and $\sigma$) are indicated. The N and C-termini of the $\alpha$ subunits are drawn. The two hexamer sequences recognized by RNA polymerase are the -10 and -35 elements and the start site for transcription is marked (+1). The -35 site is proposed to be necessary for the initial binding of RNA polymerase to the promoter and the -10 site is where nucleation of DNA strand separation occurs. The length of the DNA separating the -10 and -35 elements, designated the spacer DNA (SP), can drastically effect promoter activity. Another determinant of promoter activity is an AT rich region positioned between -40 and -60 of some promoters termed the upstream (UP) element. This sequence is contacted by the $\alpha$ subunit of RNA polymerase. At some promoters a 5' extension of the -10 element, a TG dinucleotide at positions -14 and -15, creates an alternative contact point for region 2.5 of $\sigma$ such that transcription can be initiated in the absence of a specific -35 element. Adapted from deHaseth et al., 1998.
sequence is contacted by the α subunit of RNA polymerase and its presence can increase promoter activity by as much as a factor of 30 (Frisby and Zuber, 1991; Ross et al., 1993; Frederick et al., 1995). Gourse et al. (1986) proposed that interaction of the αCTD with the UP element creates a bend in the DNA that is important for promoter activation. Additionally, at some promoters a 5' extension of the -10 element, a TG dinucleotide at positions -14 and -15 (Bown et al., 1997), creates an alternative contact point for region 2.5 of σ (see below) such that transcription can be initiated in the absence of a specific -35 element (Ponnambalam et al., 1986; Kielty and Rosenberg, 1987; Burns et al., 1996).

Promoters can contain different combinations of these DNA elements. Some promoters carry all the information required for efficient initiation of transcription by RNA polymerase alone. In others, the nucleotide sequences of promoters are deficient in information for one or more of the steps in the initiation pathway. This deficiency prevents efficient promoter utilization in the absence of a transcription activator. Activators facilitate initiation by accelerating one or more of the steps. The majority of activators function by binding neighboring DNA sites and interacting with RNA polymerase (Ishihama, 1993; Ptashne and Gann, 1997).

1.3. RNA polymerase.

The transcription of genes in bacteria and eukaryotes is executed by a multisubunit enzyme. Some RNA polymerase subunits show sequence homology between these two kingdoms (Polyakov et al., 1995), although the eukaryotic transcription apparatus is more complicated (see Ptashne and Gann, 1997 and references therein for recent review). RNA polymerase can be viewed as a molecular motor, translocating along the DNA in a process that derives energy from the catalysis of nucleotide polymerization (Gelles and
Landick, 1998). The core enzyme of bacterial RNA polymerase is a heterotetramer comprised of three different polypeptides, beta (β), beta prime (β') and alpha (α), with a stoichiometry of ββ'α2 (Figure 1). This complex exhibits non-specific DNA binding and is capable of transcript elongation. Specific promoter recognition is bestowed upon association of the sigma (σ) subunit with the core to generate the holoenzyme (ββ'α2σ). The β' subunit participates in DNA binding, the β subunit harbours the active site for nucleotide polymerization and the α subunits contribute as a scaffold for core assembly (Yura and Ishihama, 1979; Hayward et al., 1991; Ishihama, 1993; Ebright and Busby, 1995). Several dissociable subunits have also been found to associate with RNA polymerase. The delta (δ) subunit of B. subtilis RNA polymerase plays an important role in preventing polymerase association with non-specific DNA and weak promoters to increase the selectivity of transcription by RNA polymerase (Achberger and Whiteley, 1981; Achberger et al., 1982a,b; Hyde et al., 1986; Dobinson and Spiegelman, 1987; Juang and Helmann, 1994, 1995). The omega (ω) subunit of E. coli RNA polymerase has no known function (Gentry et al., 1991) but it can act as a transcription activator when fused to a DNA binding protein that tethers RNA polymerase to DNA and may provide a target for prokaryotic transcription activators (Dove and Hochschild, 1998). Both the β and β' subunits have been identified as targets for transcriptional activators as well (Miller et al., 1997; Szalewska-Palasz et al., 1998). Recent investigations have demonstrated that the α and σ subunits also play critical roles in DNA and activator contacts and these will be briefly reviewed below.

1.3.1. The α subunit.

The RNA polymerase α subunit consists of two domains connected by a flexible linker (Figure 1). The N-terminal domain of alpha (αNTD) is the platform
for assembly of the core enzyme (Ishihama, 1981; Igarashi et al., 1991a) while the C-terminal domain (αCTD) makes contacts with both the DNA (Ross et al., 1993; Frederick et al., 1995) and a variety of transcription factors (Igarashi et al., 1991b; Igarashi and Ishihama, 1991; Mencia et al., 1996; Mencia et al., 1998; see Ishihama, 1993 for review). Mutations in the E. coli rpoA gene (which encodes the RNA polymerase α subunit) localized transcription factor contact sites to the C-terminal 79 amino acids of the α subunit (Figure 2). This region of α has been designated Contact site I (Figure 3). Activators that depend on this region of α for their stimulatory effects are classified as Class I activators. The αNTD has also been shown to contain an activator contact site (Busby and Ebright, 1997).

In addition to its role as a transcription factor contact site, the alpha subunit also recognizes cis-acting DNA signals termed upstream (UP) elements (Ross et al., 1993; Frederick et al., 1995; Gaal et al., 1996; Murakami et al., 1996). UP elements enhance transcription initiation by increasing the equilibrium constant for RNA polymerase binding to DNA (Rao et al., 1994). They are generally located a few nucleotides 5' to the -35 basal element. The region of α that contacts UP elements overlaps Contact site I (Gaal et al., 1996; Murakami et al., 1996).

1.3.2. The σ subunit

Although core polymerase is capable of catalyzing RNA synthesis, specific initiation of transcription is absolutely dependent on the σ subunit. There are 4 conserved regions of amino acids among all prokaryotic σ factors, excluding those of the σ54 family (Helmann and Chamberlin, 1988). Regions 1-4 can be further subdivided into segments to which specific responsibilities in transcription can be assigned (for review see Helmann, 1988; Gross, 1992).
Figure 2. Alignment of the C-terminal amino acids of the wild type *E. coli* and *B. subtilis* α subunits of RNA polymerase. Shown is an alignment of the C-terminal 76 and 65 amino acids of the *E. coli* and *B. subtilis* α subunits, respectively. The positions of a 73 amino acid deletion in the *E. coli* alpha and of a 15 or 59 amino acid deletion in the *B. subtilis* alpha are indicated. Comparison of the sequences indicates that the 59 amino acid deletion in the *B. subtilis* alpha is equivalent to a 73 amino acid deletion in the *E. coli* alpha. Mutational mapping of the *rpoA* gene (which encodes the RNA polymerase α subunit) has localized transcription contact sites for several *E. coli* (representatives are marked above the sequences) and a *B. subtilis* regulator p4 (marked below the sequences) to the C-terminal 73 amino acids of the α subunit (for references see Introduction). Adapted from Mencia et al., 1996.
Region 1.1 modulates the DNA binding activity of σ and is also important for efficient transcription initiation (Dombroski et al., 1993). Region 1.2 also plays a role in open complex formation (Wilson and Dombroski, 1997). Region 2 has 5 segments identified to date. Segment 2.1 is involved in the binding of σ to core enzyme (Lesley and Burgess, 1989). A function has not yet been ascribed to region 2.2. Mutational analysis has demonstrated that region 2.3 participates in DNA melting (Juang and Helmann, 1994b) and the transition to an elongation complex (Jones and Moran, 1992; Jones et al., 1992). Region 2.4 interacts directly with the promoter -10 site (Siegele et al., 1989; Zuber et al., 1989; Waldburger et al., 1990), while region 2.5 is responsible for recognition of the 'extended -10' motif of promoters (Figure 1; Barne et al., 1997). The precise role of region 3 in transcription initiation is unknown.

Region 4 of σ also plays a major role in DNA recognition. A conserved helix-turn-helix recognition motif in region 4.2 interacts directly with the -35 promoter element (Figure 1; Siegele et al., 1989; Zuber et al., 1989; Waldburger et al., 1990; Dombroski et al., 1992) and adjacent amino acids are involved in protein-protein contacts with several transcription factors in E. coli and B. subtilis (Figure 2; Ishihama, 1993; Kumar et al., 1994; Li et al., 1994; Baldus et al., 1995; Kim et al., 1995; Li et al., 1997; Schyns et al., 1997; Buckner and Moran, 1998). Activators that depend on this region of σ for their stimulatory effects are classified as Class II activators (Figure 3).

2.0. Sporulation in Bacillus subtilis.

Bacterial cells constantly monitor environmental conditions for signals which dictate their metabolic state. Alterations in parameters such as cell density, temperature, nutrient status or the level of toxic compounds provide signals that trigger physiological responses by the bacteria to cope with these
Figure 3. *E. coli* activator contact regions on RNA polymerase and promoters. A, the activator contact regions on the α and σ subunits are marked. B, the double lines represent the DNA and black boxes mark the -10 and -35 sequences for polymerase recognition. The gray boxes indicate the position and number of the binding sites for the adjacent transcription factor at the promoter within the brackets. Class I activators generally bind to DNA sites upstream of the -35 region and stimulate transcription through contact site 1 of the α subunit. Class II activators tend to bind to DNA sites overlapping the -35 region and stimulate transcription through contact site 2 of the σ subunit. Representatives of each Class are shown. Adapted from Ishihama, 1993.
changes (Freese, 1981; Grossman and Losick, 1988; Trach et al, 1991; Grossman, 1995). One of the most striking examples of this adaptive ability occurs in \textit{B. subtilis} during continued starvation. Signals which indicate nutrient depletion initiate a complex series of events leading to the remarkable morphological response of endospore formation.

2.1. Induction of sporulation.

To create the endospore, the cell alters the process of cell division from a central to a polar position (Figure 4) so that two unequally sized cellular compartments are formed within one cell wall (Piggot and Coote, 1976; Errington, 1993; Stragier and Losick, 1996). The larger compartment is designated the mother cell and the smaller compartment is destined to become the forespore, with each compartment receiving a chromosome from the last round of DNA replication. Eventually, the small compartment is engulfed by the mother cell to produce a cell-within-a-cell termed a forespore. Each compartment follows a very different path of gene expression determined by the synthesis and activation of compartment specific sigma factors which associate with core RNA polymerase (Stragier and Losick, 1990; Losick and Stragier, 1992; Errington, 1993; Haldenwang, 1995; Stragier and Losick, 1996). The mother cell contributes to the maturation of the forespore, including the deposition of a proteinaceous coat and dehydration and mineralization of the core. Lysis of the mother cell 6-8 hours after the onset of sporulation releases the endospore, a 'genetic safe' that is resistant to heat, ultraviolet radiation and other environmental stresses.

The signals that initiate the sporulation response have not been completely identified. However, considerable progress has been made in determining the proteins involved in the process of transmission of the
Figure 4. The sporulation decision of *Bacillus subtilis*. When environmental conditions are conducive to rapid growth, the cell follows the path to the left and divides symmetrically to produce identical daughter cells. Alterations in parameters such as cell density, temperature, nutrient status or the levels of toxic compounds provide signals that trigger the transcription of sporulation-genes which leads to endospore formation. As shown in the scheme to the right, during endospore formation, the cell alters the process of cell division from a central to a polar position so that two unequally sized cellular compartments are formed within one cell wall. The larger compartment becomes the mother cell and the smaller compartment is destined to become the forespore. The two compartments follow different developmental paths determined by the synthesis and activation of compartment specific sigma factors (Figure 7) which associate with core RNA polymerase. The endospore can return to vegetative growth through germination and outgrowth.
starvation signals for initiating and regulating the sporulation response. Classical genetic studies have identified almost 50 loci that participate in the control of sporulation. Mutations that block sporulation were categorized according to the stage of developmental arrest. Cells arrested at the earliest stages of spore development contained mutated genes whose products were required in the production or recognition of signals that initiate the sporulation cycle. The isolation of such mutants led to the identification of the genes encoding key effectors of sporulation. Transition into the sporulation pathway is governed by a complex "two component" regulatory system (Hoch, 1993a, b).

2.2. Two-Component regulators.

The capability of bacteria to couple their physiological state to environmental conditions provides an essential selective advantage. The ability to sense-and-adapt is mediated through numerous classes of signal transduction systems that have evolved to link environmental stimuli to cellular responses. One such class is the two-component regulatory systems that are ubiquitous among bacteria (Stock, J. B. et al., 1989; Parkinson and Kofoed; Stock et al., 1995; Voltz, 1995) and even eukaryotes (Chang et al., 1993; Ota and Varshavsky, 1993). At their heart is a sensor-regulator pair and each member is comprised of modular protein domains (Stock, J. B. et al., 1989). Environmental signals are detected by the input domain of the sensor, which is often, but not always, a membrane associated kinase (Figure 5). The signal is transduced to its output domain which autophosphorylates on a conserved histidine residue. Using a mechanism conserved among the homologous regulatory pairs, the phosphate is transferred to a conserved aspartate residue in the receiver domain of the cognate response regulator. This phosphorylation
Figure 5. Two-component signal transduction systems. Two component systems are comprised of a sensor kinase-response regulator pair and each member is comprised of modular protein domains. Environmental signals (●) are detected by the input domain of the sensor kinase, which are often, but not always, membrane proteins. The signal is transduced to its output domain which autophosphorylates on a conserved histidine residue. The phosphate is transferred to a conserved aspartate residue in the N-terminal receiver domain of the cognate response regulator. This relieves inhibition of the activity of the C-terminal output domain which usually functions by binding DNA sequences within target promoters and specifically stimulating or repressing promoter activity.
relieves inhibition of the activity of the output domain of the response regulator. If the stimulus alters gene expression, then the response regulator usually functions by binding DNA sequences within target promoters and specifically stimulating or repressing promoter activity. To date, over 300 of these systems have been identified in more than 100 different bacterial genera (Hoch and Silhavy, 1995).

2.3. The phosphorelay.

The signal transduction system for the initiation of sporulation is an elaborate extension of the type described above because it involves 4 components (Figure 6). Environmental signals trigger the autophosphorylation of sensor kinase proteins (KinA, KinB or KinC). The phosphoryl group is then transferred to an aspartate residue on Spo0F, a member of the response regulator family consisting solely of the receiver domain. Spo0F~P serves as a substrate for an enzyme unique to the phosphorelay, Spo0B. Spo0B is a phosphoprotein phosphotransferase that catalyzes the transfer of the phosphoryl group from Spo0B~P to Spo0A. The pathway culminates with the phosphorylation and activation of the response regulator protein Spo0A (Burbulys et al., 1991; Hoch, 1995). Spo0A is an ambiactive transcription factor which either initiates or represses expression from specific promoters.

Spo0A is the central regulator of the sporulation response. The inability to isolate an intergenic suppressor of a Spo0A null mutation supports this concept. Spo0A~P is responsible for regulating synthesis of the sporulation specific sigma factors σE (the spollG operon in the mother cell) and σF (the spollA operon in the forespore), and several components of the phosphorelay itself (Stragier and Losick, 1990; Errington, 1993; Hoch, 1993b; Grossman, 1995; Stragier and Losick, 1996). High levels of activated Spo0A (Spo0A~P),
in response to low nutrient levels, shifts the balance in favor of endospore formation (Chibazakura et al., 1991; Ireton et al., 1993; reviewed in Grossman, 1995). The decision to initiate spore formation is a critical pivot point in the bacterial cycle because it occurs at the expense of continued growth. Cells that sporulate in the presence of adequate nutrients are at a disadvantage since they cannot proliferate and compete. Thus, the level of Spo0A-P is tightly regulated by several phosphatases which act directly on SpoOF-P or Spo0A-P (Perego et al., 1994; Perego et al., 1996; Perego and Hoch, 1996) and a recently discovered kinase inhibitor (Kipl) which prevents the transfer of phosphate from KinA to SpoOF (Wang, L. et al., 1997).

2.4. Promoters regulated by Spo0A.

Transcription regulation by Spo0A-P is complex and a number of genes are directly affected by Spo0A (reviews Hoch, 1993a,b; Hoch, 1995). For the onset of sporulation, two sets of genes are important: basic components of the phosphorelay and proteins modulating their expression, and the sigma cascade.

2.4.1. Phosphorelay components.

Spo0A, Spo0B and Spo0F are maintained at a basal level during vegetative growth. This ensures that the cells will be able to respond rapidly to changes in the environment. The spo0F and spo0A genes contain dual promoters. During vegetative growth they are transcribed constitutively from the vegetative promoters by RNA polymerase containing σA. Low levels of Spo0A-P allow for high-level production of the AbrB repressor from the abrB gene (Figure 6), since the transcription of the abrB gene is negatively regulated by direct binding of Spo0A-P to its promoter (Strauch et al., 1990; Greene and
Spiegelman, 1996). Repression of AbrB by Spo0A–P has several important consequences (reviewed in Errington, 1993; Grossman, 1995; Hoch; 1995). AbrB negatively regulates transcription from the spoOH gene, which encodes the alternative sigma factor (σH) required to transcribe the genes for several key phosphorelay components. σH-RNA polymerase directs transcription from the sporulation specific promoters of spoOF and spo0A as well as the kinA gene, which encodes for the major sporulation sensor kinase (Figure 6). Spo0A–P also positively regulates its own synthesis from the vegetative promoter of spo0A. The end result is an increase in the cellular concentration of Spo0A–P. Once levels of Spo0A–P sufficient to ensure initiation of sporulation are attained, Spo0A–P may repress further transcription of these genes. In addition, the repression of AbrB production relieves repression of the spo0E gene. Spo0E is a phosphatase that dephosphorylates Spo0A–P and stifles the flow of phosphate to Spo0A.

2.4.2. The sigma factor cascade.

Spo0A–P has promoter targets outside of the phosphorelay components. Two of these operons, spollG (Satola et al., 1991; Satola et al., 1992; Bird et al., 1993; Baldus et al., 1994) and spollA (Wu, et al., 1989 Wu et al., 1991), are positively regulated by Spo0A–P (Figure 7). The spollG operon is transcribed by σA-RNA polymerase and the spollA operon is transcribed by σH-RNA polymerase. The spollG and spollA operons encode the sporulation-specific sigma factors, σE and σF, respectively. Transcription of these two genes initiates the sigma factor cascade in the development of the endospore (Errington, 1993; Hoch, 1993a; Grossman, 1995; Haldenwang, 1995). This cascade involves the association of different sporulation-specific sigma factors with core polymerase to direct compartmentalized transcription in the forespore and mother-cell. The
Figure 6. Transcriptional interaction among genes of the phosphorelay and the initiation of the sigma factor cascade. This figure shows the flow of phosphate from the sensor kinases to the regulator, Spo0A. Thin arrows denote the direction of phosphotransfer between phosphorelay components. Thick arrows with + and - signs indicate positive or negative regulation of the target activity by the indicated protein. Genes for the phosphorelay components directly regulated by Spo0A-P and the RNA polymerase form, $\sigma^A$ or $\sigma^H$, which transcribe the promoters are indicated next to the gene. KipR negatively regulates Kipl which in turn negatively regulates KinA activity. The phosphatases Spo0L and Spo0P act directly on Spo0F-P and the Spo0E phosphatase acts on Spo0A-P. The transcription repressor, AbrB, negatively regulates $\sigma^H$ and degradative enzyme production.
sequential production of sigma factors provides a mechanism for the temporal control of gene expression during sporulation.

2.4.3. The spollG promoter.

Most of the studies pertaining to the mechanism of transcription stimulation by Spo0A have been conducted on the spollG promoter because it is transcribed by the RNA polymerase containing the major vegetative sigma factor, σA, which can be readily purified (Dobinson and Spiegelman, 1987). It has proven difficult to obtain sufficient quantities of σH-RNA polymerase for detailed in vitro analysis of the spollA promoter, although some studies on σH have been reported (Wu et al., 1989; Bird et al., 1992; Asayama et al., 1995).

Mutational analysis and in vitro footprinting of the promoter for the spollG operon demonstrate that there are two sets of tandem 0A boxes upstream of this promoter (Figure 8). The binding of Spo0A~P to one set, termed site 1, protects from -95 to -70 and binding to site 2, leads to protection from -60 to-35, respective to the start site for transcription (Satola et al., 1991; Baldus et al., 1994). The 0A boxes in site 1 (the strong binding site) are closer to the consensus recognition sequence than those in site 2 (the weak binding site), yet deletion of site 1 did not change regulation of the spollG promoter in vivo (Baldus et al., 1994). The structure of the spollG promoter is unusual in two respects. First, it lacks a consensus -35 sequence in the normal position 17-18 bp upstream of the -10 sequence. A potential -35 sequence is found 22 bp upstream, placing it out of phase with the -10 region. Second, the start site for transcription is spaced 2 nucleotides further from the -10 region than is found for most promoters transcribed by σA-RNA polymerase (Kenney and Moran, 1987; Kenney et al., 1988; Kenney et al., 1989). These features are suggested to be
the major barrier to initiation of transcription by RNA-polymerase at this promoter.

3.0. A model for SpoOA activation of the spollG promoter.

Previous in vitro studies have demonstrated that the phosphorylation of SpoOA markedly stimulates transcription from a DNA fragment carrying the spollG promoter (Satola et al., 1992; Bird et al., 1993). It was observed that Spo0A-P had an increased affinity for the 0A boxes, particularly in the weaker site 2 region (Baldus et al., 1994). Bird et al. (1996) demonstrated that RNA polymerase bound the spollG promoter in the absence of Spo0A but the complex was inefficient at initiating transcription. The addition of Spo0A led to the formation of a new complex, but transcription was not significantly stimulated. In contrast, the addition of Spo0A-P generated a complex different from that found with Spo0A which could rapidly initiate transcription. This indicated that the phosphorylation of Spo0A enhanced its ability to modify RNA-promoter complexes. During the course of this thesis work, genetic studies have shown that Spo0A stimulation is mediated through contacts with the sigma subunit of RNA polymerase (Baldus et al., 1995; Schyns et al., 1997; Buckner and Moran, 1998; Hatt and Youngman, 1998).

Spo0A is unique among transcriptional activators. It is the only positive regulator demonstrated to activate transcription from promoters transcribed by RNA polymerase containing different sigma factors (Baldus et al., 1995). Spo0A regulates transcription by RNA polymerase from both σA-dependent and σH-dependent promoters. Recently, a σA specific activation region has been identified in Spo0A (Figure 9; Buckner et al., 1998; Hatt and Youngman, 1998) and mutations in σA and σH that specifically decrease transcription from Spo0A-
dependent promoters (Baldus et al., 1995; Schyns et al., 1997; Buckner and Moran, 1998) have been isolated (covered in detail in the Discussion).

3.1. Mechanisms of activation by positive regulators.

The ease with which transcription is initiated is promoter-dependent: some promoters direct efficient initiation by RNA polymerase alone while others are dependent upon activators for expression. Considerable genetic and biochemical data indicate that activators function by binding to specific sequences within promoters and stimulate transcription by contacting RNA polymerase. In keeping with the mode of Spo0A stimulation of spolIG transcription, the studies of several transcription factors from E. coli that have laid the foundation for mechanisms of activation by positive regulators are reviewed. Attention is focused on model systems involving activator interaction with the sigma subunit of RNA polymerase to increase the rate of isomerization of the closed to open complex.


Also known as cAMP receptor protein (CRP), CAP has the capacity to interact with different subunits of holoenzyme, in a promoter dependent manner, to stimulate transcription (for reviews see (Ebright, 1993; Busby and Ebright, 1997). With as many as 10% of all E. coli genes under its control, the activity of CAP has profound effects on cell physiology and has been the subject of intense investigation. CAP activates transcription as a dimer and its DNA binding activity is modulated by cAMP. In the absence of cAMP, CAP dimers have a low affinity for DNA but they are converted to a high affinity sequence-specific DNA binding protein when complexed with cAMP. When bound, CAP dimers bend the DNA greater than 90° (Bracco et al., 1986; Liu-Johnson et al.,
Figure 7. The sigma factor cascade and compartmentalized gene expression. Spo0A-P is a transcription activator of the spollG and spollA operons which are transcribed by RNA polymerase containing σ^A and σ^H, respectively. The σ^E and σ^F products are responsible for the transcription of genes whose products are used exclusively in the developing forespore (σ^K) and mother cell (σ^G). Adapted from Hoch, 1993.
1986; Busby, 1992) thereby promoting interaction of RNA polymerase with DNA upstream of the CAP binding sites (Gartenberg and Crothers, 1991; Schultz et al., 1991).

The RNA polymerase subunit that CAP dimers contact and the stage of initiation that is stimulated is related to the location of the CAP binding site within the promoter. For example, at the lac promoter CAP behaves as a Class I transcription activator binding distal to the -35 site (Ishihama, 1993) and stimulating the initial binding of RNA polymerase to the promoter through contacts with the C-terminal domain of the α subunit (αCTD; Malan et al., 1984; Igarashi and Ishihama, 1991; Ebright, 1993 and references therein). The region in CAP used for these contacts has been designated AR1.

At the gal promoter, CAP behaves as a Class II transcription activator (Ishihama, 1993), binding a region that overlaps the -35 site and stimulating both RNA polymerase recruitment to the promoter and isomerization of closed to open complex through contacts with both the αCTD and the N-terminal domain of the α subunit (αNTD; Bell et al., 1990a; Bell et al., 1990b; Busby and Ebright, 1997 and references therein). Stimulation of transcription of Class II promoters by CAP is dependent upon AR1 and a second activating region designated AR2.

The interaction of CAP dimers with RNA polymerase is not restricted to the α subunit. An additional de novo activation region (AR3) was created by a single amino acid substitution in CAP (Bell et al., 1990a; Williams et al., 1991; West et al., 1993; Niu et al., 1996). This mutation specifically enhanced transcription from Class II dependent promoters and partially suppressed the effects of substitutions in AR1 and AR2 at Class II promoters. Remarkably, the suppression arises from establishment of a new CAP contact with region 4 of σ70. However, AR3 is a non-native activation region. A natural sigma contact
region for CAP has been proposed by Kumar and colleagues (1994) who, by way of deletion analysis, suggest that CAP may interact with region 3 of $\sigma^{70}$.

3.1.2. Lambda repressor ($\lambda$cl)

Another classic model for transcription activation is phage lambda repressor which governs expression from divergent promoters ($P_R$ and $P_{RM}$) in the OR (operator right) region. Transcription from the promoters is controlled by the binding of $\lambda$cl to three 17 bp recognition sites, OR1, OR2, and OR3. Occupation of OR1 and OR2, which overlap $P_R$, by $\lambda$cl dimers represses that promoter and prevents expression of genes required for the lytic cycle. In this arrangement, the repressor dimer bound at OR2 activates transcription of its own gene from $P_{RM}$, the promoter for synthesis of repressor required for maintenance of lysogeny (Ptashne, 1992). Elevated levels of repressor lead to binding at OR3 and repression of both promoters. Mutations which specifically destroy the activation function of $\lambda$cl (positive control mutations) have been isolated and cluster on the surface of the protein in a region predicted to be in close proximity to RNA polymerase when bound at the promoter (Guarente et al., 1982; Hochschild et al., 1983; Bushman et al., 1989). The RNA polymerase target of this activating region in $\lambda$cl has been genetically localized to region 4 of $\sigma^{70}$ (Kuldell and Hochschild, 1994; Li et al., 1994). $\lambda$cl has been shown to stimulate the isomerization of closed to open complexes without having much affect on RNA polymerase binding to the promoter (Hawley and McClure, 1982).

3.1.3. PhoB

Under conditions of phosphate limitation the PhoB protein of *E. coli* activates expression of an operon governing the transport and assimilation of phosphate (Nakata et al., 1987). PhoB is a response regulator that is
phosphorylated by its cognate sensor kinase, PhoR (Makino et al., 1989). PhoB is a monomer in solution but dimerizes upon phosphorylation, activating its DNA binding activity (Fiedler and Weiss, 1995; McCleary, 1996). N-terminal deletion mutants of PhoB bind to specific sequences (Pho box) without the need for phosphorylation (Makino et al., 1996). Deletion analysis of σ^70 suggests that PhoB stimulates transcription through contacts with the sigma subunit (Makino et al., 1993; Kumar et al., 1994).

The RNA polymerase interaction domain of PhoB has been localized to a region proposed to form a helix-loop-helix DNA recognition motif analogous to that of OmpR (Pratt and Silhavy, 1994; Kondo et al., 1997; Martinez-Hackert and Stock, 1997a,b). This variant of the classical helix-turn-helix motif (Harrison and Aggarwal, 1990; Harrison, 1991) carries extra residues between the two helices that interact with the DNA, creating a loop rather than a turn. The proximity of this loop to the DNA binding motif serves to place this region in the appropriate position for interaction with sigma when holoenzyme and the activator are both bound at the promoter.

There are four PhoB boxes at the pstS promoter spaced 11 bp apart, so that if each bound one PhoB protein, the 4 would be aligned along one face of the DNA helix. Insertion of the recognition helix of PhoB into the major groove of the DNA would induce DNA bending to produce DNA arched around a central PhoB complex (Makino et al., 1996). The σ^70 -10 basal element is 15 bp downstream of the most proximal PhoB box. A PhoB-RNA polymerase interaction would require that their respective binding sites be in phase, thus the intervening DNA is predicted to be untwisted. Much of the model for PhoB activation remains speculative; it is not known which PhoB boxes are absolutely required for activation, whether DNA untwisting is a necessary component of the mechanism or which step in the initiation pathway is affected by PhoB–P.
Figure 8. Features of the spollG promoter. The linear sequence of the non-template strand (A) and the predicted three-dimensional configuration (B) are shown. At the spollG promoter the -10 and -35 RNA polymerase consensus sequences (A, shaded; B, black dots) are separated by 22 bp instead of the usual 17 to 18 bp, placing them on opposite faces of the DNA helix. The Spo0A boxes (A, overlined; B, arrows) occur in tandem and comprise the site 1 and site 2 OA binding sites. A stretch of 5 adenine nucleotides between the OA sites is presumed to generate a bend in the DNA centred at position -68. Nucleotide positions are numbered relative to the transcription start site (A, •; B, $\text{r}^\triangleright$). H, HindIII; As, Asel; Al, Alul.
A

H

AAGCTTATCGACAAATTAAGCAGATTTCCCTGAAAAATTGTA

-100 -90 -80 -70

TTTTCCTCTCAACATTAA

-60 -50 -40 -30 -20 -10 +1

As

Al

B

site 2

-100

-80

-60 -40 -20 +1

site 1
3.1.4. Nitrogen regulatory protein C (NtrC).

NtrC is a transcriptional activator for several σ^{54}-dependent promoters in *E. coli* and *Salmonella* (Kustu et al., 1989). NtrC is the response regulatory partner of the NtrB kinase (Parkinson and Kofoid, 1992) and activates transcription in response to nitrogen limitation (reviewed in Porter et al., 1995). The activity of NtrC is regulated by phosphorylation (Ninfa and Magasanik, 1986; Keener and Kustu, 1988; Weiss and Magasanik, 1988).

The target sequences for NtrC~P behave like eukaryotic enhancers, *i.e.* they can function even when displaced several hundred base pairs from their original position (Reitzer and Magasanik, 1986; Ninfa et al., 1987). Phosphorylation enhances the DNA binding activity of NtrC. This has several important consequences: i) it tethers the activator near regulated promoters (Wedel et al., 1990); ii) it stimulates oligomerization of NtrC~P dimers (Wyman et al., 1997); iii) it induces an ATPase activity (Mettke et al., 1995; Wedel and Kustu, 1995; North et al., 1996). Each function is absolutely required for transcriptional activation by NtrC. NtrC~P contacts σ^{54}-RNA polymerase through formation of a DNA loop (Su et al., 1990) and stimulates isomerization of closed to open complexes (Ninfa et al., 1987; Su et al., 1990; Wedel et al., 1990).

Biochemical analysis of open complex formation revealed at least two points in the initiation pathway at which NtrC figures prominently; i) it overcomes a kinetic barrier by inducing a conformational change in σ^{54}-holoenzyme; ii) it removes a thermodynamic barrier by stabilizing a complex containing separated DNA strands. The two-step activation model begins with NtrC mediated disruption of a patch of leucine residues in the N-terminus of σ^{54} that derepresses the melting activity of holoenzyme containing σ^{54} (Wang et al., 1995; Wang and Gralla, 1996; Syed and Gralla, 1997). The open complexes
formed by disruption of the leucine patch are unstable, suggesting they are in dynamic equilibrium with closed complexes.

In the second stage, the open complex is stabilized through protein-protein contacts and the hydrolysis of ATP (Wedel and Kustu, 1995; Wang, J. T. et al., 1997). The mechanism for coupling ATP hydrolysis to stable open complex formation has not yet been elucidated. The rate-limiting step in this process is not related to denaturation of the DNA strands since transcription from pre-opened promoter templates did not bypass the requirement for NtrC or ATP-hydrolysis. Rather, the focal point of NtrC function appears to be modification of the activity of σ^{54} (Wedel and Kustu, 1995). However, a 10% increase in transcription by RNA polymerase alone from the bubble templates over duplex DNA indicated that DNA denaturation partially suppressed the activator requirement. Furthermore, deletion of the N-terminus of σ^{54} (which negatively regulates its DNA melting function), mutation of the -12 basal element (necessary for repression of transcription in the absence of NtrC) or reaction conditions that favor DNA melting (low salt and elevated temperature) can bypass the enhancer requirement of σ^{54}-holoenzyme (Wang, J. T. et al., 1997). Each of these conditions serves to unlock the DNA strand melting property of σ^{54}-holoenzyme. Thus the stimulation of DNA strand separation is an important regulatory feature of NtrC.

The unusual characteristics of σ^{54} make it difficult to ascertain the general applicability of this activation scheme to activators outside the NtrC class. The sequence of all known sigma factors are homologous (Helmann and Chamberlin, 1988; Lonetto et al., 1992) with the exception of the σ^{54} class, which bears scant resemblance (Merrick, 1993). The stringent positional requirements of the conserved -12 and -24 dinucleotides, which must be exactly one helical turn apart (10 bp), distinguishes this class of σ factor from the rest
whose conserved promoter elements are normally separated by 2 to $2^{1/2}$ turns of DNA (Ray et al., 1990; Gralla and Collado-Vides, 1996).

3.2. The characteristics of Spo0A.

Although the 3-D structure of Spo0A is unknown, the amino acid sequence of its N-terminal domain is very similar to that of CheY, a response regulator involved in regulating bacterial chemotaxis in *Escherichia coli* (Stock, J. B. et al., 1989; Stock and Surette, 1995). The crystal structure of CheY has been solved (Stock, A. M. et al., 1989; Voltz and Matsumura, 1991; Zhu et al., 1997a). The N-terminus is comprised of five $\alpha$-helices and a five stranded parallel $\beta$-sheet, in a $(\beta/\alpha)_5$ topology. The $\beta$-sheet forms a central core surrounded by three $\alpha$-helices on one face and two on the opposite side to create a filled barrel. Phosphorylation of Spo0A occurs on a conserved aspartate residue, D56, in the N-terminus of the protein (Figure 9).

The crystal structures of the C-terminus of OmpR (Kondo et al., 1997; Martinez-Hackert and Stock, 1997a, b) and NarL (Baikalov et al., 1996) provide some insight into the organization of the C-terminal domain of response regulators. The C-terminal DNA-binding domains of OmpR and NarL are comprised of a compact bundle of 3 and 4 $\alpha$-helices, respectively, of which two form the helix-turn-helix (HTH) recognition motif (Harrison and Aggarwal, 1990; Harrison, 1991). A probable HTH DNA binding motif has been identified in the C-terminus of Spo0A (Brown et al., 1994) and a DNA consensus sequence, a Spo0A box, for Spo0A-P binding has been determined with the sequence 5' TGNCGAA 3', where N is usually a T nucleotide (Strauch et al., 1990; Baldus et al., 1994).

The N-terminal and C-terminal domains of Spo0A are connected by a flexible linker; they can be separated by trypsin cleavage (Grimsley et al., 1994).
Figure 9. Map of the activating regions of Spo0A. The Spo0A protein consists of two domains: the N-terminal phosphoacceptor domain and the C-terminal output domain are joined by a flexible linker. Phosphorylation occurs on a conserved aspartate residue (D56) within the N-terminus. The trypsin cleavage site is marked.

Mutations in Spo0A which specifically decrease activation of $\sigma^A$-dependent promoters ($\sigma^A$-R1) are indicated (Buckner et al., 1998; Hatt and Youngman, 1988). Also indicated are the A257V and A257E amino acid substitutions of the spoA9V and spo153 mutants, respectively. $\sigma^A$-R2 marks a potential $\sigma^H$ activation region. The positions of the two spo0A9V suppressor mutants, $suv3$ (L174F) and $suv4$ (H162R) are also indicated. Mutations in Spo0A which affect transcription from spollG described in this work are in bold. The horizontal bar denotes the proposed HTH DNA-binding domain. Adapted from Hatt and Youngman, 1998.
The isolated N-terminus can be phosphorylated in vitro and the C-terminal fragment, termed the Spo0A binding domain (Spo0ABD), generates a DNase I footprint at the abrB promoter that is indistinguishable from the footprint of wild type protein (Strauch et al., 1990). Furthermore, N-terminal deletion mutants of Spo0A are constitutively active in vivo (Ireton et al., 1993) and in vitro (Grimsley et al., 1994). This suggests that the two domains can fold and function independently of one another. Clearly, the C-terminal fragment of Spo0A is capable of recognizing the appropriate DNA binding sequences to effect transcription and the N-terminal fragment is a negative regulatory domain which keeps Spo0A in the inactive state. This is a common regulatory feature of many response regulators.

4.0. Experimental rationale.

The initial binding of RNA polymerase to a promoter depends on sequence-specific interactions between the -10 and -35 elements of the promoter and the sigma subunit of RNA polymerase (Gross et al., 1992). However, the subsequent steps of transcription initiation are not well understood. In addition, positive control transcription factors can bind specific DNA sequences in target promoters and interact with RNA polymerase to activate transcription. Considerable insight into the contributions of discrete domains within transcription activators has expanded our previously limited understanding of regulator function. However, the mechanism by which transcription is initiated through the concerted actions of RNA polymerase and activators is still poorly defined. This study was initiated to detail the mechanism of transcription stimulation by the response regulator Spo0A at the spollG promoter. The aim was to advance the understanding of the general mechanism of activator function.
Using a potent *in vitro* system of recombinant proteins, the activity of Spo0A at the *spolIG* promoter was examined in the hopes of answering several fundamental questions regarding transcription stimulation by Spo0A-P. For example, what role does the higher DNA binding affinity of phosphorylated Spo0A play in activation? What specific functions of activation can be ascribed to the N- and C-termini of Spo0A? What is the molecular mechanism underlying Spo0A-P activity? Are separate domains within Spo0A required for stimulation of σ^A_ and σ^H_ dependent promoters? Are there additional regions in Spo0A required for stimulation of *PspolIG*? Does Spo0A also interact with the C-terminus of the α subunit of RNA polymerase to stimulate the *spolIG* promoter? To directly address these mechanistic questions, a constitutively active deletion mutant of Spo0A comprised of the C-terminal 124 amino acids of the protein was characterized alongside the unmodified and phosphorylated forms of Spo0A. The Spo0A model was refined, with emphasis on the mechanism underlying gene activation at the *spolIG* promoter.
EXPERIMENTAL PROCEDURES.

1.0. Bacterial strains and media.

Bacterial strains used in this work are listed in Table 3. Luria-Bertani (LB; Sambrook et al., 1989) and Schaeffer’s Sporulation Media (SSM; Schaeffer et al., 1965) contained the appropriate antibiotics at the following final concentrations: chloramphenicol, 5 μg/ml; kanamycin, 2 μg/ml; ampicillin, 100 μg/ml. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in dimethylformamide was used at a final concentration of 75 μg/ml in indicator agar. Standard genetic techniques, enzymatic reactions and DNA manipulations were conducted as described (Sambrook et al., 1989) or as recommended by the manufacturer. Bacterial transformations for E. coli (Hanahan et al., 1991) and B. subtilis (Hoch, 1991) were done as described.

2.1. Wild type spolIG template DNA.

The spolIG template was isolated from the plasmid pUCIIGtrpA (Satola et al., 1991) on a 600 bp DNA fragment generated by digestion with PvuII. This fragment contained the spolIG promoter, both upstream Spo0A binding sites and the trpA transcription terminator approximately 160 bp downstream from the transcription start site. The trpA terminator functioned effectively in vitro. The fragment was electroeluted from agarose gel slices and stored in 10 mM Hepes, 20 mM potassium acetate, 0.1 mM EDTA, pH 8.0 at 4 °C. The concentration of template fragment was determined from A260nm.

2.2. RNA polymerase purification.

B. subtilis RNA polymerase was isolated as described (Dobinson and Spiegelman, 1987). Preparations were free of RNAse and DNAse and judged to be >98% pure by Coomassie blue staining of sodium dodecyl
sulfate/polyacrylamide gels. The polymerase concentration was determined using the Micro BCA (bicinchoninic acid) assay using bovine serum albumin (Fraction V, Sigma Chemical Co.) as a standard. The activity of the preparations was determined using single round transcription assays from the Spo0A-independent G2 promoter under DNA saturating conditions (Bird, 1995). Typically, preparations were found to be 30 to 50% active.

Polymerases reconstituted with the mutant alpha subunits (a gift of M. Salas, Universidad Autónoma) were prepared and assayed on the bacteriophage φ29 AC2 promoter (Mencia et al., 1996; Monsalve et al., 1996a). The specific activities of the wild type and mutant holoenzymes were similar. Equivalent amounts of the reconstituted polymerases were added separately to single round in vitro transcription reactions (Bird et al., 1993).

2.3. Phosphorylation of Spo0A.

Spo0A, and the proteins required to phosphorylate it, were a gift of J. A. Hoch (Scripps Research Institute). The phosphorylation of Spo0A was carried out as previously described (Bird et al., 1993). Briefly, phosphorylation reactions contained 8.0 µM Spo0A and 3.2 µM each of SpoOB and Spo0F and 0.32 µM KinA in 1x transcription buffer (20 µl final volume). The reaction was initiated by the addition of ATP to 1 mM and the sample was incubated at room temperature for 90 min. A constant volume of appropriately diluted reaction mixture (in 1x transcription buffer) was added to transcription reactions. Samples of unphosphorylated Spo0A or Spo0ABD did not contain KinA to ensure against low level phosphorylation during transcription initiation reactions.
2.4. *In vitro* transcription assays.

Standard transcription reactions (20 μl final volume) contained as final concentrations: 4.0 nM template, 80 mM potassium acetate, 0.4 mM each of ATP, UTP and CTP (FPLC grade, Pharmacia Biochemicals), 0.005 mM [α-\(^{32}\)P]GTP (800 Ci/mmol; New England Nuclear (NEN)) in 1x transcription buffer (40 mM Hepes (pH 8.0), 5 mM MgAc, 0.1 mM EDTA, 0.1 mM DTT and 0.1 mg/ml BSA; Bird *et al.*, 1996). A 2 μl aliquot of an appropriate dilution of either Spo0A, Spo0A–P or Spo0ABD was added to transcription samples containing template DNA, potassium acetate and the initiating nucleotides ATP and GTP in a 14 μl volume. Following a 2 minute incubation at 37°C (to allow binary complex formation), transcription was initiated by adding 2 μl of RNA polymerase (diluted in 1x transcription buffer containing 30% glycerol (v/v)). The reactions were incubated a further 2 minutes to allow the formation of initiated polymerase-promoter complexes and RNA elongation was permitted by the addition of 2 μl of elongation mix (UTP, CTP and heparin (10 μg/ml final concentration)). After 5 minutes, reactions were stopped by the addition of one-fifth the volume loading buffer (8.0 M urea, 180 mM Trizma base, 180 mM boric acid, 4 mM EDTA, 0.02% each bromophenol blue and xylene cyanole).

Radio-labelled transcripts were separated from unincorporated nucleotide by electrophoresis through a 6% polyacrylamide gel containing 7 M urea (Sambrook *et al.*, 1989). Labelled transcripts were localized by autoradiography using XAR film (Kodak Co.) and promoter activity was determined by measuring the Cerenkov radiation in excised gel slices that contained the transcripts or on a Molecular Dynamics Phospholmager SI using Imagequant software (v. 1.0). The number of RNA transcripts was calculated by measuring the specific activity of the GTP in the labelling mix either as Cerenkov radiation or pixel count in a 2 μl sample of the labelling mix.
Multiplying this value by 36 (the number of GTPs per full length spollG transcript) provided a measure of the specific activity of transcripts produced for each reaction. Transcription activity was determined as the percentage of templates yielding an RNA transcript (Bird, 1995). Salt stability experiments report the type and concentration added to the assays.

3.0. Primer extension analysis.

Primer extension was used to map the start site of transcription as described earlier (Bird et al., 1993). DEPC treated water (0.1% (v/v)) was used for primer extension analysis. RNA from single-round transcription reactions was recovered from polyacrylamide gel slices by electroelution. 10 μg of yeast tRNA (in DPEC treated water) were added and the samples were extracted twice with phenol/chloroform and the RNA was precipitated with ethanol (Sambrook et al., 1989). The RNA was dissolved in 7 μl of water and 2 μl of 5x hybridization buffer (250 mM Tris pH 8.3, 500 mM KCl) and 1 μl (2 pmol) of IIG2 primer (Table 1) end-labelled with T4 polynucleotide kinase and [γ-32P]ATP (7000 Ci/mmol; ICN Biochemicals) were added. Hybridization was conducted at 90°C for 1 min, 50°C for 2 min and then ice for 15 min. Next, 6 μl of 5x avian myeloblastosis virus reverse transcriptase buffer (AMVRT; Promega), 0.5 μl RNAsin (an RNase inhibitor), 0.5 μl AMVRT, 4 μl dNTP mix (10 mM dATP, dCTP, dGTP, dUTP) were added and the volume brought to 30 μl with water. The mixture was incubated for 1 hr at 42°C before addition of 1 μl EDTA (0.5 M, pH 8.0) and 2 μl RNase A (10 mg/ml). Incubation was continued for 15 min at 37°C and the extension products were recovered by adding 4 μl sodium acetate (3 M, pH 5.2) and 120 μl ethanol and incubating at -70°C for 1 hr. Products were collected by centrifugation for 15 min at 4°C. The spollG gene was sequenced with the end-labelled IIG2 primer and pUCIIGtrpA as template using a United
States Biochemicals (USB) sequencing kit. The products of the extension and sequencing reactions were electrophoresed through an 8% polyacrylamide gel containing 7 M urea.

4.0. KMnO₄ footprint reactions.

Template DNA was prepared as described by Bird et al. (1993). The plasmid pUCIIGtrpA (15 μg) was digested with 20 units of BamH I (which cuts 135 bp downstream of the transcription start site) in a final volume of 20 μl for 1 hr at 37°C. The DNA was treated with phenol/chloroform, precipitated with ethanol and dissolved in 18 μl of calf intestinal alkaline phosphatase buffer (CIAP; Boheringer Mannheim). 2 μl of CIAP were added and the sample incubated at 37°C for 2 hr. SDS and EDTA (0.5 M, pH 8.0) were added to final concentrations of 0.5% and 5 mM, respectively, followed by a 30 min incubation at 65°C. The DNA was treated with phenol/chloroform, precipitated with ethanol and dissolved in 24 μl of sterile distilled water. 2 μl of [γ-³²P]ATP (7000 Ci/mmol; ICN Biochemicals), 1 μl T4 polynucleotide kinase (10 units/μl; Gibco) and 3 μl kinase forward reaction buffer were added and the reaction incubated at 37°C for 30 min. The reaction was then incubated at 65°C for 15 min to inactivate the kinase. The sample was cooled, brought to a final volume of 60 μl in 1x PvuII reaction buffer and PvuII (40 units) was added. Following a 1hr incubation at 37°C, the labelled fragments were separated on a 4% polyacrylamide gel and the 410 bp fragment containing the promoter was extracted by electroelution. The efficiency of end-labelling was determined by measuring the Cerenkov radiation in a diluted sample. DNA with a specific activity of 1.5 x10⁷ cpm/μg was routinely obtained.

Standard transcription reactions were prepared as described containing a final concentration of 4 nM labelled-DNA (10⁵ cpm). KMnO₄ modifications
were carried out by the addition of 1 μl of a freshly prepared 200 mM KMnO₄ solution. After three minutes at 37°C, the reaction was terminated by the addition of 50 μl of stop solution (1.5 M 2-mercaptoethanol, 0.1 mM EDTA, 0.36 M sodium acetate (pH 7.0), 120 μg/ml sonicated salmon testis DNA). The samples were precipitated with ethanol, dissolved in 100 μl of 1 M piperidine, incubated 30 minutes at 90°C and placed on ice. Samples were precipitated with butanol (Sambrook et al., 1989) and residual piperidine was removed by vacuum centrifugation for 30 minutes (Savant centrifuge). DNA was redissolved in 5 μl of formamide loading buffer (Sambrook et al., 1989) and heated at 90°C for two minutes before loading onto an 8% polyacrylamide sequencing gel containing 7 M urea. An equal number of cpm from each reaction (10⁵ cpm) was loaded in each lane. The gels were dried and exposed to X-ray film.

5.0. DNase I footprint reactions.

End-labelled template DNA was prepared as described for KMnO₄ reactions above. DNase I (added to a final concentration of 0.67 mg/ml; Sigma Chemical Co.) reactions were performed in 1X transcription buffer at 37°C for 10 s (Bird et al., 1996) and terminated by the addition of 75 μl stop buffer (0.1% SDS, 4 mM EDTA, 270 mM NaCl and 40 μg/ml sonicated calf thymus DNA (Sigma Chemical Co.)). Samples were precipitated with ethanol and dissolved in 5 μl of formamide loading buffer (Sambrook et al., 1989) and heated at 90°C for two minutes before loading onto an 8% polyacrylamide sequencing gel containing 7 M urea. An equal number of cpm from each reaction (10⁵ cpm) was loaded per lane. The gels were dried and exposed to X-ray film. Measurement of the specific binding for SpoOA, Spo0A–P and Spo0ABD was determined from autoradiographs of the DNase I protection patterns of the three
activator forms at the *spollG* promoter. Autoradiographs were scanned at 10 bits, gray scale using an AGFA Scann II scanner. Protection of the site 1 and 2 SpoA boxes was assessed by measuring the intensity of $^{32}$P-labelled bands within the protected regions and normalizing them to the intensity of bands outside the protected regions on a Power Macintosh (6100/66) using a Molecular Dynamics Phospholmager SI and Imagequant software (v. 1.0).

6.0. Electrophoretic mobility shift assays (EMSA).

End-labelled DNA was prepared as described for the KMnO$_4$ footprint reactions except that the second digestion was performed with HindIII to give a 230 bp fragment. Reactions contained template DNA (10$^4$ cpm), RNA polymerase (40 nM) and either SpoOA, Spo0A-P or Spo0ABD at a final concentration of 400 nM in 1x transcription buffer in a final volume of 10 µl. The initiating nucleotides ATP and GTP were at 0.4 mM final concentrations when present. Samples were incubated at 37°C for 3 minutes to allow ternary complex formation before the addition of one-third the volume of loading buffer containing heparin (10 µg/ml heparin, 20% glycerol (v/v) in 1x transcription buffer) or, where indicated, loading buffer containing calf thymus DNA (0.3 mg/ml sonicated calf thymus DNA, 20% glycerol (v/v) in 1x transcription buffer). Samples were immediately loaded onto a running (20 ma) 4.5% acrylamide gel containing 40 mM acetic acid, 40 mM Trizma base, 1 mM EDTA and 2% glycerol. Electrophoresis was continued for 2 hours, the gels were dried and exposed to x-ray film.

7.0. Mutation of the *spollG* promoter.

The parent plasmid, pUClIGtrpA (Bird *et al.*, 1993) contains a 240 bp HindIII to BamHI DNA fragment bearing the *spollG* promoter and 100 bp 5' to
the transcription start site, in which both the 0A binding sites reside. Two 35-mer oligonucleotide primers, MB1 and MB2 (Table 1), were purchased from the Nucleic Acid and Protein Service Unit (University of British Columbia). These primers were complementary to the template strand from -26 to +8 with respect to the transcription start site and included the AluI site (Figure 10A), except each primer contained a stretch of 8 or 12 nucleotides identical, rather than complementary, to the template strand from -7 to -14 or -3 to -14. PCR products were generated using Taq Polymerase (Boehringer Mannheim), pUCIIgtrpA as a template and a downstream primer (IIG2; Table 1), which anneals to the coding strand adjacent to the BamHI site.

The PCR products were cloned into the pGEM-T PCR cloning vector (Promega) to create pDR8 and pDR12 (Table 2). Inserts were verified by the dideoxy chain termination sequencing method using the Sequenase 2.0 DNA sequencing system (U. S. Biochemicals). The HindIII-AluI fragment which contains the two 0A binding sites, was isolated from pUCIIgtrpA and ligated to the AluI-BamHI fragment isolated from pDR8 or pDR12 to reconstitute the full length promoter. The ligation mixture was used as template for a PCR reaction using the downstream primer described above and an upstream primer (IIGA, Table 1), complementary to the template strand which annealed adjacent to the HindIII site. The resulting PCR product was cloned into pGEM-T to create pDR8IIG and pDR12IIG (Table 2). Inserts were verified as described above. The E. coli strain DH5α (Table 3) was used for all of the necessary transformations.


The plasmids pUCIIgtrpA, pDR8IIG and pDR12IIG were digested with HindIII and BamHI. The 235 bp promoter bearing fragments were isolated by
Table 1. Mutagenic primers used in mutational PCR of the *spoIIIG* promoter and the *spo0A* gene.

<table>
<thead>
<tr>
<th>Primers&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sequence&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mutation Introduced</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>spoIIIG</em> primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MB1</td>
<td>CAGAGCTTGCTTATATGAATACTTTGCAAGAAAGG</td>
<td>8 nt denatured region</td>
</tr>
<tr>
<td>MB2</td>
<td>CAGAGCTTGCTTATATGAATTTGCAAGAAAGG</td>
<td>12 nt denatured region</td>
</tr>
<tr>
<td>IIGA</td>
<td>CCAAGCTTATCGACAAATTA</td>
<td>upstream IIG primer</td>
</tr>
<tr>
<td>IIG2</td>
<td>GGGGATCCTCTAGAGTCA</td>
<td>downstream IIG primer</td>
</tr>
<tr>
<td><em>spo0A</em> primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HindIII0A</td>
<td>GGAAGCTTTTGGGGAGGAAGAAGG</td>
<td>upstream 0A primer</td>
</tr>
<tr>
<td>0A4</td>
<td>CGGGATCCAAAGACGTTTGAT</td>
<td>downstream 0A primers</td>
</tr>
<tr>
<td>0AΔT</td>
<td>CCGGATCCTTAAAGACCTTATGCTCTA</td>
<td>deletion of terminator</td>
</tr>
<tr>
<td>0AΔ10</td>
<td>CCGGATCCTTACGCAACCATT</td>
<td>deletes last 10 residues</td>
</tr>
<tr>
<td>0AΔ15</td>
<td>CCGGATCCTTAAATTCCTCG</td>
<td>deletes last 15 residues</td>
</tr>
<tr>
<td>0AV258</td>
<td>TTAAGAGCCCTATGCTCTAACCCTACGTTAACC</td>
<td>valine at 258 of Spo0A</td>
</tr>
<tr>
<td>0AV259</td>
<td>TTAAGAGCCCTATGCTCTAACCCTACGACATCCGC</td>
<td>valine at 259 of Spo0A</td>
</tr>
<tr>
<td>0AV260</td>
<td>TTAAGAGCCCTATGCTCTACCTACCTACCTGCC</td>
<td>valine at 260 of Spo0A</td>
</tr>
<tr>
<td>0AV261</td>
<td>TTAAGAGCCCTATGCTCTACACCAGCTT</td>
<td>valine at 261 of Spo0A</td>
</tr>
<tr>
<td>0AV262</td>
<td>TTAAGAGCCCTATGCTCTACCCCTCA</td>
<td>valine at 262 of Spo0A</td>
</tr>
<tr>
<td>0AV263</td>
<td>TTAAGAGCCCTATGCACTAACCCTCAG</td>
<td>valine at 263 of Spo0A</td>
</tr>
<tr>
<td>0AV264</td>
<td>TTAAGAGCCCTTACCTCACC</td>
<td>valine at 264 of Spo0A</td>
</tr>
<tr>
<td>0AV265</td>
<td>TTAAGAGCCACATGCTC</td>
<td>valine at 265 of Spo0A</td>
</tr>
<tr>
<td>0AV266</td>
<td>TTAAGAGCCCTTATGCTC</td>
<td>valine at 266 of Spo0A</td>
</tr>
<tr>
<td>0AV267</td>
<td>TTAAGAGCCCTTATGCTC</td>
<td>valine at 267 of Spo0A</td>
</tr>
</tbody>
</table>

<sup>a</sup>HindIII0A and 0A4 were gifts of J. Middlekamp and M. Cervin.

<sup>b</sup>Primers are written left to right in the 5' to 3' direction; nucleotides altered to encode a valine in the corresponding position of Spo0A are underlined.
Table 2. Plasmids used in subsequent studies.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUCIIGtrpA</td>
<td>pUC19 with a 240 bp HindIII to BamHI DNA fragment bearing the spoIIG promoter and 100 bp 5' to the transcription start site with the trpA transcription terminator.</td>
<td>Satola et al., 1991</td>
</tr>
<tr>
<td>pGEM-T</td>
<td>Ampicillin based vector for cloning of PCR products.</td>
<td>Promega</td>
</tr>
<tr>
<td>pBluescript SK(+-)</td>
<td>DNA production</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pDR8</td>
<td>pGEM-T harbouring a mutated Alul to BamHI fragment of the spoIIG promoter with an inverted stretch of 8 nts within the -10 region</td>
<td>This study</td>
</tr>
<tr>
<td>pDR12</td>
<td>pGEM-T harbouring a mutated Alul to BamHI fragment of the spoIIG promoter with an inverted stretch of 12 nts within the -10 region</td>
<td>This study</td>
</tr>
<tr>
<td>pDR8IIG</td>
<td>The HindIII-Alul fragment of spoIIG ligated to the Alul-BamHI fragment from pDR8 in pGEM-T</td>
<td>This study</td>
</tr>
<tr>
<td>pDR12IIG</td>
<td>The HindIII-Alul fragment of spoIIG ligated to the Alul-BamHI fragment from pDR12 in pGEM-T</td>
<td>This study</td>
</tr>
<tr>
<td>pSKWTIIIG+</td>
<td>The wild type HindIII to BamHI fragment of spoIIG in pBluescript SK+</td>
<td>This study</td>
</tr>
<tr>
<td>pSKWTIIIG-</td>
<td>The wild type HindIII to BamHI fragment of spoIIG in pBluescript SK-</td>
<td>This study</td>
</tr>
<tr>
<td>pSK8IIG+</td>
<td>The HindIII to BamHI fragment of pDR8IIG in pBluescript SK+</td>
<td>This study</td>
</tr>
<tr>
<td>pSK8IIG-</td>
<td>The HindIII to BamHI fragment of pDR8IIG in pBluescript SK-</td>
<td>This study</td>
</tr>
<tr>
<td>pSK12IIG+</td>
<td>The HindIII to BamHI fragment of pDR12IIG in pBluescript SK+</td>
<td>This study</td>
</tr>
<tr>
<td>pSK12IIG-</td>
<td>The HindIII to BamHI fragment of pDR12IIG in pBluescript SK-</td>
<td>This study</td>
</tr>
<tr>
<td>pJM103</td>
<td>pUC19 with a chloramphenicol cassette functional in B. subtilis.</td>
<td>Perego, 1993</td>
</tr>
<tr>
<td>pDRM1</td>
<td>spo0A9V in pJH103</td>
<td>This study</td>
</tr>
<tr>
<td>pDRM2</td>
<td>spo0A153 in pJH103</td>
<td>This study</td>
</tr>
<tr>
<td>pDRM3</td>
<td>spo0AΔT in pJH103</td>
<td>This study</td>
</tr>
<tr>
<td>pDRM4</td>
<td>spo0AΔ10 in pJH103</td>
<td>This study</td>
</tr>
<tr>
<td>pDRM5</td>
<td>spo0AΔ15 in pJH103</td>
<td>This study</td>
</tr>
<tr>
<td>pDRM6</td>
<td>spo0AV258 in pJH103</td>
<td>This study</td>
</tr>
<tr>
<td>pDRM7</td>
<td>spo0AV259 in pJH103</td>
<td>This study</td>
</tr>
<tr>
<td>pDRM8</td>
<td>spo0AV260 in pJH103</td>
<td>This study</td>
</tr>
<tr>
<td>pDRM9</td>
<td>spo0AV261 in pJH103</td>
<td>This study</td>
</tr>
<tr>
<td>pDRM10</td>
<td>spo0AV262 in pJH103</td>
<td>This study</td>
</tr>
<tr>
<td>pDRM11</td>
<td>spo0AV263 in pJH103</td>
<td>This study</td>
</tr>
<tr>
<td>pDRM12</td>
<td>spo0AV264 in pJH103</td>
<td>This study</td>
</tr>
<tr>
<td>pDRM13</td>
<td>spo0AV265 in pJH103</td>
<td>This study</td>
</tr>
<tr>
<td>pDRM14</td>
<td>spo0AV266 in pJH103</td>
<td>This study</td>
</tr>
<tr>
<td>pDRM15</td>
<td>spo0AV267 in pJH103</td>
<td>This study</td>
</tr>
</tbody>
</table>
electroelution from agarose gel slices and ligated into the pBluescript SK+ and SK- vectors (Stratagene) digested with the same enzymes to produce pSK8IIG+, pSK8IIG-, pSK12IIG+, pSK12IIG-, pSKWTIIG+ and pSKWTIIG- (Figure 10A; Table 2). *E. coli* JM101 cells (Table 3) containing pBluescript plasmids bearing the desired inserts were grown on minimal medium plates to select for the presence of the F episome. A single colony was used to inoculate 5 ml of 2x YT medium containing ampicillin and the culture was incubated overnight at 37°C with shaking (180 rpm). 1 ml of the overnight culture was used to inoculate 50 ml of 2x YT containing ampicillin in a 250 ml flask. The culture was incubated 30 min at 37°C with vigorous agitation. Single-stranded DNAs were generated by infection of the cells with M13K07 helper phage at a multiplicity of infection of 20 pfu per cell. Incubation was continued at 37°C for 30 min, kanamycin was added to 70 μg/ml and the cells were incubated overnight with shaking. Cells were removed by centrifugation for 15 min at 17,000 rpm, the supernatant retained and centrifuged again. The phage particles were collected by adding 1/4 the volume of a 20% polyethyleneglycol (PEG 8,000), 3.5 M ammonium acetate solution to the supernatants. The samples were mixed gently by inversion, and placed on ice for 30 min. The phage particles were collected by centrifugation at 14,000 rpm for 15 min. The pellets were resuspended in TE, extracted extensively with phenol/chloroform, treated with RNaseA and the DNA precipitated with ethanol (Sambrook *et al.*, 1989). The DNA was dissolved in 20 μl of distilled water and stored at -20°C.

To produce heteroduplex templates, complementary single-strands (10 μg each) were mixed and adjusted to 0.2 N NaOH in a final volume of 10 μl. Samples were heated at 37°C for 15 minutes, neutralized with HCl, brought to a final volume of 27 μl in 1x *PvuII* reaction buffer and incubated a further 15 minutes. *PvuII* (30 units) was added and digestion allowed to proceed for 2 hrs
Figure 10. Creation of the heteroduplex templates. (A) Top box, the *spolIG* promoter sequence is shown with the site 2 0A boxes marked by the black bars and the -10 and -35 sequences marked by the white bars. Oligonucleotide primers (MB1/MB2) containing either 8 or 12 nucleotides of template sequence corresponding to the -10 region of the promoter (-7 to -14 or -3 to -14, respectively) were used in a PCR reaction with the IIG2 primer to mutate the *spolIG* promoter sequence as described in the *Experimental Procedures*. After ligating the mutated region to the wild type upstream region of the promoter, the PCR products were cloned into the PCR cloning vector pGEM-T and sequenced. Middle box, mutant (*) promoters were excised from pGEM-T clones by digestion with HindIII and BamHI and cloned into pBluescript SK(+)/- digested with the same enzymes. Bottom box, single stranded DNAs of mutant and wild type promoters were produced by infection of the *E. coli* clones with helper phage and annealed with the complementary wild type or mutant strands to produce double-stranded DNA containing the desired region of heteroduplex. (B) Agarose gel of a representative heteroduplex template annealing reaction. M, double stranded DNA markers with sizes to the left shown in base pairs. DNA isolated following phage infection of *E. coli* clones carrying the complementary pSK12G + and - single strand DNAs were tested by digestion with *PvuII*. A 685 bp product was not observed, confirming the DNA was single strand. The two were mixed and treated with NaOH and HCl as described in the *Experimental Procedures*. Digestion of this DNA with *PvuII* produced the expected 685 bp heteroduplex template.
B
+ - + pSK12G+
- + + pSK12G-
- - + NaOH/HCl

M + + + Pvull

2726 —
1818 —
1272 —
727 —
600 —
Figure 11. Structures of heteroduplex templates. The non-complementary regions of heteroduplex templates used in the subsequent transcription assays are shown. Mismatch bubbles (MB) of 8 or 12 base pairs in length were created containing the non-template sequence (NT) or the template sequence (T) of the -10 site (overlined) within the non-complementary region. Not shown is the wild type template produced by annealing the wild type (+) and (-) DNA strands. The transcription start site (+1) and the -10 positions are marked.
at 37°C. The promoter bearing PvuII fragments (675 bp; Figure 10B) were purified on an agarose gel, electroeluted and ethanol precipitated. The templates (MB8T, MB8NT, MB12T and MB12NT) are shown in Figure 11 and described in Table 4.

8.0. Mutation of the spo0A gene.

The upstream and downstream primers, HindIII0A and 0A4 (Table 1), were used to amplify the spo0A9V and spo0A153 mutants from chromosomal DNA isolated from B. subtilis strains JH695 and JH994 (Table 3). The 880 bp PCR products were cloned into pGEM-T. Site-specific mutations were introduced into the spo0A gene by PCR amplification using the upstream HindIII0A primer and one of the mutagenic downstream primers listed in Table 1 with the plasmid pKK0A (which harbours the full length wild type spo0A gene; J. Hoch, Scripps Research Institute) as template. The mutated products were cloned into pGEM-T as recommended by the manufacturer (Figure 12). The sequence of all spo0A alleles were verified (Nucleic Acid and Protein Service Unit, University of British Columbia). Plasmid DNAs were cut at unique Sphl and Ssrl sites and the corresponding spo0A gene fragments cloned into pJM103 (Perego, 1993) digested with the same enzymes (Figure 13) to create the pDRM* series (Table 2).

8.1. Screening for sporulation defective mutants of Spo0A.

The pDRM plasmids carrying the spo0A mutants and a chloramphenicol-resistance gene were used to transform the Bacillus subtilis strain JH16304 (J. Hoch, Scripps Research Institute) to chloramphenicol resistance by Campbell-type recombination into the chromosomal spo0A locus. JH16304 harbours a PspollG-lacZ transcriptional fusion linked to a kanamycin-resistance marker integrated ectopically at the amyE locus (J. A. Hoch, Scripps...
Figure 12. Mutation and subcloning of the spo0A gene. A, site-specific mutations were introduced into the spo0A gene by PCR amplification using the upstream (U) HindIII OA primer and one of the mutagenic (*) downstream (D) primers listed in Table 1. The plasmid pKK0A, which harbours the full length wild type spo0A gene, was used as template. The mutated products were cloned into pGEM-T. The upstream and downstream primers, HindIII OA and 0A4, were used to amplify the spo0A9V and spo0A153 alleles from chromosomal DNA isolated from B. subtilis strains JH695 and JH994 (Table 3). The 880 bp PCR products were cloned into pGEM-T. The sequences of all spo0A alleles were verified.
Figure 13. Transformation of mutant spo0A alleles into *B. subtilis*. The plasmid DNA (Figure 12) was restricted at unique *SphI* and *SstI* sites and the corresponding *spo0A* gene fragments cloned into pJM103 digested with the same enzymes to create the pDRM series. The pDRM plasmids carrying the *spo0A* mutants (*) were used to transform the *Bacillus subtilis* strain JH16304 to chloramphenicol resistance by Campbell-type recombination into the chromosomal *spo0A* locus. The T7 promoter associated with the plasmid does not function in *Bacillus*.
Table 3. Bacterial strains used in subsequent studies.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Descriptiona</th>
<th>Reference/ Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>[hsdR17(rK&lt;sup&gt;−&lt;/sup&gt;mK&lt;sup&gt;+&lt;/sup&gt;) supE44 thi-1 rexA1 gyrA (Nal&lt;sup&gt;r&lt;/sup&gt;) relA1 Δ(lacZYA-argF)U169 (φ80lacZΔM15)]</td>
<td>Gibco</td>
</tr>
<tr>
<td>JM101</td>
<td>[F' trad36 proA&lt;sup&gt;+&lt;/sup&gt; proB&lt;sup&gt;+&lt;/sup&gt; lacIq lacZΔM15/supE thi Δ(lac-proAB)]</td>
<td>Gibco</td>
</tr>
<tr>
<td><strong>B. subtilis</strong>b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JH642</td>
<td>trpC2 phe-1</td>
<td>J. Hoch</td>
</tr>
<tr>
<td>JH695</td>
<td>spo0A9V</td>
<td>Ferrari et al., 1985</td>
</tr>
<tr>
<td>JH994</td>
<td>spo0A153</td>
<td>Ferrari et al., 1985</td>
</tr>
<tr>
<td>JH16304</td>
<td>amyE::spolIG-lacZ, Kn&lt;sup&gt;R&lt;/sup&gt;</td>
<td>J. Hoch</td>
</tr>
<tr>
<td>DR2001</td>
<td>JH16304ΩpDRM1</td>
<td>This study</td>
</tr>
<tr>
<td>DR2002</td>
<td>JH16304ΩpDRM2</td>
<td>This study</td>
</tr>
<tr>
<td>DR2003</td>
<td>JH16304ΩpDRM3</td>
<td>This study</td>
</tr>
<tr>
<td>DR2004</td>
<td>JH16304ΩpDRM4</td>
<td>This study</td>
</tr>
<tr>
<td>DR2005</td>
<td>JH16304ΩpDRM5</td>
<td>This study</td>
</tr>
<tr>
<td>DR2006</td>
<td>JH16304ΩpDRM6</td>
<td>This study</td>
</tr>
<tr>
<td>DR2007</td>
<td>JH16304ΩpDRM7</td>
<td>This study</td>
</tr>
<tr>
<td>DR2008</td>
<td>JH16304ΩpDRM8</td>
<td>This study</td>
</tr>
<tr>
<td>DR2009</td>
<td>JH16304ΩpDRM9</td>
<td>This study</td>
</tr>
<tr>
<td>DR2010</td>
<td>JH16304ΩpDRM10</td>
<td>This study</td>
</tr>
<tr>
<td>DR2011</td>
<td>JH16304ΩpDRM11</td>
<td>This study</td>
</tr>
<tr>
<td>DR2012</td>
<td>JH16304ΩpDRM12</td>
<td>This study</td>
</tr>
<tr>
<td>DR2013</td>
<td>JH16304ΩpDRM13</td>
<td>This study</td>
</tr>
<tr>
<td>DR2014</td>
<td>JH16304ΩpDRM14</td>
<td>This study</td>
</tr>
<tr>
<td>DR2015</td>
<td>JH16304ΩpDRM15</td>
<td>This study</td>
</tr>
</tbody>
</table>

a: ; spolIG-lacZ is integrated in the amyE gene; Ω, plasmid is integrated in the chromosome; Kn<sup>R</sup>, kanamycin resistance gene.
b: all B. subtilis strains were derived from the parent JH642 and contain the trpC2 and phe-1 mutations.
Research Institute). Transformants were selected by plating on LB containing chloramphenicol and kanamycin. Ten representatives from each plate were chosen and their ability to sporulate was determined by plating on SSM plates containing the appropriate antibiotics. The *B. subtilis* mutant strains created in this study are listed in Table 3.

8.2. Determination of the sporulation frequency.

Selected sporulation deficient (spo<sup>+</sup>) transformants were streaked on LB plates with antibiotic and grown at 37°C for 24 hr. A single colony was resuspended in 5 ml SSM with antibiotic and incubated with shaking (180 rpm) for 24 hr. The total number of viable cells per ml was determined by serial dilution and plating on LB agar containing kanamycin and chloramphenicol. The number of spores per ml was determined by adding 0.1 volume of chloroform to an aliquot of cultured cells (Dartois *et al.*, 1996) and plating appropriate dilutions as described above. The sporulation frequency was calculated as the ratio of spores to total number of viable cells. Expression of the promoter fusions was determined by β-galactosidase assay (see below) and the repressor function of Spo0A mutants was monitored by streaking of selected transformants on milk agar plates with antibiotic (Higerd *et al.*, 1972).

8.3. β-galactosidase assays.

The *PspollG-lacZ* promoter fusion was used as a reporter of Spo0A stimulation function in the Spo0A mutant transformants. β-galactosidase assays were conducted as described by Ferrari *et al.*, (1986). Briefly, LB broth containing antibiotic was inoculated with the wild type and mutant *B. subtilis* strains carrying the *spollG-lacZ* fusion and cultured overnight at 37°C with shaking (180 rpm). Samples were diluted 100x into 25 ml of SSM containing
kanamycin and chloramphenicol and the incubation continued. At various times during logarithmic growth and stationary phase, 1 ml samples were withdrawn and placed on ice for 20 min. The $A_{525nm}$ was determined and the samples were adjusted to a reading of 0.5 in a final volume of 1 ml. The cells were pelleted and stored at -70°C until assayed. To assay, pellets were resuspended in 1 ml of Z buffer (60 mM Na$_2$HPO$_4$, 40 mM NaH$_2$PO$_4$, 10 mM KCl, 1 mM MgSO$_4$ and 50 mM β-Mercaptoethanol) and placed on ice. 0.5 ml of each suspension was adjusted to a final volume of 730 µl with Z buffer, 10 µl of freshly prepared lysozyme (10 mg/ml) was added and the samples were incubated at 37°C for 20 min. 10 µl of Triton X-100 (10% (v/v)) and 100 µl of o-nitro-phenyl-galactopyranoside (ONPG; 4.5 mg/ml) were added, the samples mixed and incubated at 28°C for 15 min. Reactions were terminated by the addition of 150 µl of Na$_2$CO$_3$ (1.2 M). The $A_{420nm}$ was determined and the β-galactosidase activity in Miller units (Miller, 1972) was calculated.
RESULTS

1.0. Characterizing Spo0ABD.

The overall goal of this work was examination of the mechanism of transcription activation at the spollG promoter by Spo0A~P. One of the difficulties in working with Spo0A~P is that even the highly efficient *in vitro* phosphorelay reaction yielded only a maximum of 50% phosphorylation of Spo0A. It was known that the C-terminal fragment of Spo0A (Spo0ABD), liberated by protease treatment, was active in transcription and thus bypassed the need for phosphorylation (Grimsley *et al.*, 1994). This active form of Spo0A was useful since it eliminated the complication of the extent of phosphorylation and the carryover of ATP from phosphorelay reactions. However, only a limited characterization of transcription stimulation by Spo0ABD had been carried out (Grimsley *et al.*, 1994). To justify using Spo0ABD to study the reaction mechanism for Spo0A~P, a more detailed comparison of their characteristics was necessary and is described below.

1.1. Heparin-resistant complex formation at *spollG*.

Heparin is a potent, polyanionic competitor of protein-DNA associations (Walter *et al.*, 1967) that has been widely used for *in vitro* transcription studies. Because of its ability to quickly sequester RNA polymerase that is not stably bound, it precludes polymerase that is bound to non-specific sites on the DNA from releasing and finding a specific site (a promoter) and forming stable closed complexes. Thus, reactions can be limited to a single round when heparin is added before completion of the promoter release step. Furthermore, the number of transcripts synthesized in a single round assay is equal to the number of heparin resistant complexes formed at the promoter before heparin addition. However, heparin is invasive and is able to bind and dissociate RNA
polymerase bound specifically at some promoters (Dobinson and Spiegelman, 1987; Whipple and Sonenshein, 1992). Earlier studies showed that in the presence of SpoOA~P, B. subtilis polymerase did not attain heparin resistance at PspollG even if ATP, which allows the potential formation of the dinucleotide pppApA, was added (Figure 8; Bird, 1995). Heparin resistance was achieved if both ATP and GTP, which allows formation of an 11-mer transcript, were present. Thus, heparin-resistant complex formation at PspollG is dependent on synthesis of a transcript between 2 and 11 bases in length (Bird, 1995).

To test whether heparin resistance was attained upon synthesis of a trimer, a synthetic dinucleotide, pApA, together with GTP was added to initiation reactions to permit formation of a trimer. The lack of a 5' triphosphate on the dinucleotide prevented its incorporation into any position in the nascent transcript other than the first two bases. Transcription reactions containing Spo0ABD, RNA polymerase and the template were set up in the absence of nucleotide or under conditions that would allow formation of a dimer (ATP), a trimer (pApA and GTP) or an 11-mer transcript (Figure 14A). Conditions which permitted formation of no more than the dimer pApA did not allow the formation of ternary complexes that could survive heparin challenge and no full length transcript was produced. Complexes initiated with pApA and GTP or ATP and GTP generated full length transcripts, however, the level of heparin resistant complexes produced under pApA and GTP initiation conditions was 50-60% less than under ATP and GTP initiation conditions (Figure 14B). Nevertheless, this indicated that heparin resistant complexes accumulated under conditions that allowed synthesis of a 3 base RNA transcript.
Figure 14. Heparin-resistant complex formation at the spoIIG promoter. A, transcription reactions containing 4 nM template DNA, 400 nM Spo0ABD and 40 nM RNA polymerase were initiated with different combinations of nucleotides (as indicated above each lane). Complexes were then challenged by the addition of a mixture of heparin and the remaining nucleotides. Products were analyzed on a 6% polyacrylamide gel containing 7 M urea and the gel was exposed to x-ray film. B, the amount of transcript produced under the various initiation conditions was determined by counting the Cerenkov radiation in excised gel slices corresponding to the bands from the gel in A.
A

+ + + + RNAP
+ + + + Spo0ABD
- + - + ATP
- - + + GTP
- - + - pApA

B

![Bar chart](image)

- ATP
- pApA, GTP
- ATP, GTP

Relative percent heparin-resistant complexes
1.2. Comparison of the stimulatory properties of SpoOA, Spo0A~P and Spo0ABD.

The characteristics of stimulation of transcription by the three forms of the activator were examined to determine the differences between SpoOA and the two activated forms, Spo0A~P and Spo0ABD, and between the activated forms themselves. The results are described below.

1.2.1. Stimulation of heparin-resistant complex formation measured by transcript production in single round assays.

As a first step in examining transcription activation by Spo0ABD, stimulation of the \textit{in vitro} formation of heparin-resistant complexes by Spo0ABD, Spo0A and Spo0A~P was compared by analyzing the amount of full length transcript produced from transcription reactions initiated with ATP and GTP (see Experimental Procedures). The three forms of Spo0A were incubated with RNA polymerase and template in standard transcription assays to form initiation complexes and a single round of transcription was permitted by addition of a mixture of heparin, to inhibit further initiation, and UTP and CTP, to allow resistant complexes to elongate (Figure 15).

Spo0A was capable of slight stimulation but at 600 nM there was no more than two fold stimulation. In contrast, Spo0A~P and Spo0ABD stimulated transcription nearly 10 fold at 600 nM protein. Transcription stimulation by Spo0A~P reached a maximum level by 400 nM. At low concentrations Spo0ABD appeared to be less stimulatory than Spo0A~P; however, at higher Spo0ABD inputs, the level of initiated complexes formed was equivalent to that formed when Spo0A~P was added. These results indicated that activation of Spo0A by phosphorylation or deletion of the N-terminal domain stimulated heparin-resistant complex formation at \textit{PspollG}. 
1.2.2. Stimulation of heparin-resistant complex formation measured by electrophoretic mobility shift assay (EMSA).

Any of several steps of initiation can be influenced by a transcription activator (for review see Ptashne and Gann, 1997). One technique for distinguishing whether RNA polymerase recruitment to the promoter is the main target for the activator is electrophoretic mobility shift assays. As a first step, EMSA was used to assess the formation of heparin resistant complexes by RNA polymerase and the three forms of Spo0A (Figure 16). Mixtures of RNA polymerase, the *spollG* *HinDIII*-BamHI fragment labelled at the BamHI site, ATP and GTP, with or without one of the forms of Spo0A, were incubated for 3 min. Heparin loading buffer was added and the resistant complexes were separated by electrophoresis as described in the Experimental Procedures. The addition of RNA polymerase to the *spollG* template resulted in a small amount of DNA being retarded. This paralleled the low baseline transcription in vitro. None of the forms of Spo0A alone formed an association with the promoter which was stable to heparin challenge under the conditions used. While only a slight increase in retarded band intensity was observed when Spo0A was added to the polymerase-DNA mixture, the intensity of the shifted complex increased dramatically in samples containing Spo0A-P or Spo0ABD and RNA polymerase, in agreement with the transcription results. As seen with the transcription assays, there was little difference between the level of complexes formed with Spo0ABD and Spo0A-P. Thus, the C-terminal fragment of Spo0A contained the minimal machinery necessary to stimulate transcription from *PspollG* and the N-terminus of Spo0A was not required for formation of a transcriptionally active complex.

To test the effect of Spo0A on binding of RNA polymerase to the promoter, mobility shift assays of uninitiated complexes were conducted using
calf-thymus DNA, a non-invasive inhibitor of protein-DNA interactions (Figure 17). Under these conditions, a low level of RNA polymerase-promoter complex was observed and the addition of any of the activator forms generated only a minor increase in the amount of shifted product (lanes without ATP/GTP). When ATP and GTP were included in the binding reactions, addition of RNA polymerase alone generated a small amount of complex with a slower mobility than the complex formed without initiating nucleotides. The addition of Spo0A had no effect on the amount of this slower moving product but activated Spo0A stimulated its accumulation significantly. This stimulation paralleled the effect of activated Spo0A seen in Figure 15. The inability of Spo0A, Spo0A~P or Spo0ABD to form a stable association with the promoter on their own (Figure 16) or to increase the level of uninitiated RNA polymerase-promoter complexes (Figure 17) argued against stimulation of polymerase recruitment as the point of activator function. These results confirm previous reports from our laboratory (Bird et al., 1996) that activated Spo0A stimulates a post-recruitment step. Furthermore, Spo0ABD and Spo0A~P behaved virtually identically in these assays.

1.2.3. Stimulation of the rates of transcription

The data in Figure 15 indicated that deletion of the N-terminus of Spo0A did not reduce the maximum level of stimulation of transcription from the spolIG promoter. However, the difference in stimulation between Spo0A~P and Spo0ABD at low protein concentrations suggested that N-terminal deletion might compromise the ability of Spo0ABD to stimulate initiation in some
Figure 15. *In vitro* stimulation of transcription by Spo0A--P and Spo0ABD from *PspolIG*. Aliquots from serial dilutions of a complete phosphorelay reaction (Spo0A-P, (○)) or a reaction that lacked both KinA and ATP (Spo0A, (△) or Spo0ABD (●)) were added to transcription assays containing 80 mM potassium acetate, the initiating nucleotides ATP and GTP and template DNA. Samples were incubated for 2 min at 37°C and transcription was initiated by the addition of RNA polymerase. After 2 min, a mixture of UTP, CTP and heparin was added to block initiation but allow elongation of nascent transcripts. Transcriptional activity (expressed as the percentage of templates transcribed) was determined as a function of the total activator concentration. Error bars represent the standard deviation from three independent trials.
Figure 16. EMSA assay of Spo0A, Spo0A-P and Spo0ABD stimulation of heparin-resistant complex formation. A, Reactions containing the HindIII-BamHI fragment from pUCIIGtrpA labelled at the BamHI site, 400 nM activator alone or with RNA polymerase (indicated above each lane) were adjusted to 80 mM potassium acetate and included the initiating nucleotides ATP and GTP. Following a 3 minute incubation at 37°C, loading buffer containing heparin was added and the samples were immediately loaded onto a running 4.5% non-denaturing polyacrylamide gel. Electrophoresis was continued for 2 hours at 20 milliamps to separate complexed from free DNA. Gels were dried and exposed to x-ray film. The positions of the free DNA and shifted complexes are shown on the left.
<table>
<thead>
<tr>
<th></th>
<th>RNAP</th>
<th>Spo0A</th>
<th>Spo0A~P</th>
<th>Spo0ABD</th>
<th>ATP/GTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

shifted complex →

free DNA →
Figure 17. EMSA assay of uninitiated complex formation by Spo0A, Spo0A-P and Spo0ABD. Reactions were prepared as described in Figure 16 B except that loading buffer containing calf thymus DNA was added to the initiated (plus ATP/GTP) or uninitiated (no ATP/GTP) samples before loading. Electrophoresis was continued for 2 hours at 20 miliamps to separate complexed from free DNA. Gels were dried and exposed to x-ray film. The positions of the free DNA and shifted complexes are shown on the left.
<table>
<thead>
<tr>
<th></th>
<th>RNAP</th>
<th>Spo0A</th>
<th>Spo0A~P</th>
<th>Spo0ABD</th>
<th>ATP/GTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>shifted complex</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>free DNA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
manner. As a first test, the initial rates of initiation were measured by
determining the kinetics of heparin-resistant complex formation in reactions
stimulated by Spo0A, Spo0A-P or Spo0ABD.

Initiation reactions were composed containing template DNA, one of the
forms of Spo0A and the initiation nucleotides ATP and GTP. Reactions were
initiated by the addition of RNA polymerase and at various times samples were
removed and added to a mixture of UTP, CTP and heparin to inhibit uninitiated
RNA polymerase, but allow elongation by polymerase that had initiated RNA
synthesis. In the presence of Spo0A, the initial rate of heparin-resistant
complex formation was very low; however, the level of complexes continued to
increase with extended incubation times up to 300 sec (Figure 18A). This
indicated that Spo0A was ineffective at overcoming the rate limiting step in
heparin-resistant complex formation, although it could catalyze the transition to
heparin resistance slowly. While both the final levels and the initial rate of
formation were increased by Spo0A-P, the effect on the initial rate was most
dramatic, in agreement with the results of Bird et al. (1993). In the presence of
Spo0ABD, the final level of open complexes formed was the same as with
Spo0A-P, but a lag in the initial rate of complex formation was consistently
observed (Figure 18B). With Spo0A-P, the increase in accumulation of
heparin-resistant complexes was immediate, while reactions with Spo0ABD
required 40 to 60 s to achieve the same level of transcription complex formation
seen with Spo0A-P. Maximum levels of initiation were reached after 180 to
300 sec. The lower stimulation of the initial rate by Spo0ABD suggested that
the phosphorylated N-terminus of Spo0A-P might be involved in stabilization of
the transcription complex and/or increasing the affinity of Spo0A for the OA
boxes (see below).
1.2.4. Temperature dependence of \textit{spollG} transcription initiation.

If the observed difference between Spo0A\textsubscript{−}P and Spo0ABD in stimulation of the initial rates of formation of heparin resistant complexes reflected an enthalpic barrier, the temperature dependence of \textit{spollG} transcription stimulation by the two forms of SpoOA might differ. To test this possibility, transcription reactions containing 400 nM SpoOA, Spo0A\textsubscript{−}P or Spo0ABD were carried out at temperatures ranging from 0-42°C (Figure 19). Initiation reactions containing template DNA, RNA polymerase, one of the activator forms and ATP and GTP were composed on ice and then incubated at the indicated reaction temperature for 2 min. A mixture of heparin, UTP and CTP was added and elongation was allowed for 5 min.

Transcription in the presence of Spo0A remained baseline below 30°C and a minor increase in activity was seen at 42°C. The temperature dependence profiles for transcription stimulated by Spo0A\textsubscript{−}P and Spo0ABD were identical. Both forms of Spo0A stimulated transcription to the same extent at each of the temperatures tested. Transcription activity was detected at 25°C and increased linearly with increasing temperature. This suggested that the presence of a phosphorylated N-terminus did not affect the rate-limiting temperature-dependent step necessary for transcription from \textit{PspollG}. It was possible that the low level of transcription below 30°C was due to reduced elongation rather than a failure to form a heparin-resistant complex. To eliminate the possibility that the temperature profile represented lower elongation, the experiment was repeated, forming the DNA-activator-RNA polymerase ternary complexes at the indicated temperatures and then shifting the reactions to 37°C after addition of heparin, UTP and CTP for the elongation step. These assays yielded plots virtually identical to those above, indicating elongation was not rate limiting. The temperature curve data indicated
Figure 18. Kinetics of heparin resistant complex formation. Reactions containing 400 nM Spo0A-P (●), Spo0ABD (▲), or Spo0A (○), the spoIIIG template and the initiating nucleotides ATP and GTP were prepared. After a 2 minute incubation at 37°C, RNA polymerase was added (time zero) and at the indicated times samples were withdrawn and added to elongation mix (UTP, CTP and heparin) prewarmed at 37°C. Elongation was allowed to proceed for 5 min before the reactions were terminated and the level of transcriptional activity determined. A, a 15 minute time course with promoter activity plotted as a function of time. The dotted box is expanded in B.
Figure 19. Temperature dependence of transcription from \textit{PspollG}.
Standard transcription reactions were prepared containing 400 nM \textit{Spo0A} (▲), \textit{Spo0A-P} (●), or \textit{Spo0ABD} (○), the \textit{spollG} template and the initiating nucleotides ATP and GTP. Single round transcriptions were carried out at the indicated temperatures. Promoter activity, determined as percent templates transcribed, was plotted as a function of temperature.
that the lag in transcription initiation seen with Spo0ABD was not due to a major alteration required for initiation such as melting of the DNA strands or a conformational change in RNA polymerase.

1.2.5. Effect of anions on transcription stimulation.

The stability of protein-DNA and protein-protein complexes is strongly dependent on the reaction conditions, including the concentration and type of salt present. Cations interfere with protein-DNA interactions since protein binding requires the displacement of the cations from the polyanionic phosphate backbone of DNA, an enthalpically unfavorable process. In addition, certain anions, such as Cl\(^-\), bind to proteins with low affinities of about 10\(^{-2}\) M. These types of anions can compete with the DNA for positively charged regions on the protein. In contrast, at low concentration, ions such as acetate and glutamate do not compete with DNA but can interfere with protein-protein interactions (Arakawa and Timasheff, 1984a,b; Leirmo et al., 1987; Ha et al., 1992). Since anions can be used as probes to monitor protein-DNA and protein-protein interactions, the effects of several salts on Spo0A-dependent stimulation were tested (Figure 20). Transcription reactions were composed containing the spollG template, one of the three activator forms and the initiating nucleotides ATP and GTP. Reactions were then adjusted to the indicated salt concentration before the addition of RNA polymerase. After a 2 min incubation at 37\(^{\circ}\)C, a mixture of heparin, UTP and CTP was added and elongation allowed to proceed for 5 min. RNA transcripts were detected as described in the Experimental Procedures.

Stimulation of transcription from PspollG by Spo0A or Spo0ABD was inhibited by KCl above 40 mM. Stimulation of transcription by Spo0A\(\sim\)P was only somewhat less sensitive and was still inhibited above 80 mM KCl. Similar
inhibition profiles were seen with NaCl. Transcription stimulation was less sensitive to the presence of the organic salt potassium acetate (KAc).

Stimulation by Spo0A or Spo0ABD was not inhibited until the organic salt concentration was above 80 mM and stimulation by Spo0A~P was resistant to at least 120 mM KAc. Potassium glutamate was tested as well and was found to inhibit transcription stimulation by Spo0A and Spo0ABD, but not Spo0A~P. These results were in agreement with those of Bird (1995). A comparison of the effects of anions on transcription indicated that the phosphorylated form of the activator possessed some feature, which both Spo0A and Spo0ABD lacked, that increased resistance to acetate and glutamate, but not the inorganic ion chloride.

Chloride anion concentrations above 40 mM markedly inhibited transcription when added to reactions prior to the addition of RNA polymerase. However, when added to reactions after RNA polymerase had initiated, no inhibition of transcription was observed even at concentrations of 500 mM NaCl (Bird, 1995). Thus, the differential effect of acetate versus chloride anion on transcription stimulation by the three forms of Spo0A could be explained by the stability of the uninitiated complexes formed in their presence. To test the stability directly, initiation reactions containing the spollG promoter DNA fragment, RNA polymerase and Spo0A, Spo0A~P or Spo0ABD were formed in 120 mM KAc on ice and then warmed to 37°C. After a 1 min equilibration, aliquots were removed at various times and added to a mixture of ATP and GTP to allow initiation. After a further 2 min, a mixture of UTP, CTP and heparin was added to allow complexes which remained stable to complete a single round of elongation (Figure 21). The zero time-point was the level of transcript produced after a 1 min equilibration when KAc was added simultaneously with ATP and GTP. Transcription by RNA polymerase alone was baseline throughout the
assay. Transcription from complexes formed with Spo0A or Spo0ABD dropped to baseline within 0.5 min, while complexes containing Spo0A~P decayed slowly.

The stability of these complexes was also examined by EMSA. Reactions containing the template, RNA polymerase and one of the three activator forms were assembled on ice in the presence of 120 mM KAc. After a 3 min equilibration at 37°C, loading buffer containing calf thymus DNA was added and the samples were loaded onto a non-denaturing polyacrylamide gel as described in the Experimental Procedures (Figure 22). When initiating NTPs were omitted from reactions containing 120 mM KAc, only the Spo0A~P/RNA polymerase sample gave rise to a shifted complex. Thus, both Spo0A~P and Spo0ABD could activate transcription, but the phosphorylated N-terminus was required to stabilize the uninitiated transcription complex.

1.2.6. DNA binding by Spo0A, Spo0A~P and Spo0ABD.

The spolIG promoter contains two 0A binding sites, each with two 0A consensus binding sequences (0A boxes, Baldus et al., 1994). Site 1 (-95 to -70) contains 0A boxes that are closer to the consensus recognition sequence than those in site 2 (-60 to-35). Consequently, site 1 has been deemed a strong binding site and site 2, a weak binding site (Baldus et al., 1994). One model for the effect of phosphorylation on Spo0A is that it removes N-terminal inhibition of the C-terminal helix-turn-helix (HTH) DNA binding motif, allowing DNA binding and thus stimulation (Satola et al., 1992; Baldus et al., 1994). If this were the case, then deletion of the N-terminus of Spo0A, as in Spo0ABD, should allow it to bind uncontested to target sequences and stimulate transcription. In contrast, the apparent lower stability of complexes containing Spo0ABD relative to those
Figure 20. Influence of anion type and concentration on transcription from *PspollG*. Transcription reactions contained 100 nM Spo0A-P (●), Spo0ABD (○) or Spo0A (▲), template DNA and the initiating nucleotides ATP and GTP at the indicated salt concentrations in transcription buffer. Reactions were initiated by the addition of RNAP and a single round of transcription was permitted. Radio-labelled transcripts were separated by electrophoresis as described in the Experimental Procedures and localized by autoradiography. Promoter activity relative to that in the absence of added salt for each reaction was determined by scanning of the autoradiograph and quantification of the transcripts on a Molecular Dynamics Phospholmager SI using Imagequant software (v. 1.0). Activity was plotted as a function of salt concentration.
Figure 21. A phosphorylated N-terminus contributes to stable ternary complex formation. Transcription assays contained 400 nM Spo0A-P (○), Spo0ABD (●), SpoonA (▲) or RNA polymerase alone (△), template DNA, potassium acetate (final concentration 120 mM) and RNA polymerase. At the indicated times, 16 μl aliquots were removed and added to 2 μl of initiation mix (2 μCi [α-32P]GTP, 0.2 mM GTP and 0.4 mM ATP) prewarmed to 37°C. After a 2 minute incubation, 2 μl of elongation mix were added and the reactions were terminated after 5 min. The percent activity was plotted as a function of time.
Figure 22. Salt-stable complex formation by Spo0A-P. Transcription reactions were conducted as described in Figure 21 with the exception that the samples were adjusted to 120 mM potassium acetate and incubated at 37°C for 1 minute prior to the addition of ATP and GTP. The incubation was continued for another 3 min before loading buffer containing heparin was added. The samples were immediately loaded onto a running 4.5% non-denaturing polyacrylamide gel. Electrophoresis was continued for 2 hours at 20 milliamps to separate complexed from free DNA. Gels were dried and exposed to x-ray film. The positions of the free DNA and shifted complexes are shown on the left.
containing Spo0A–P might be due to reduced binding of Spo0ABD to DNA. To measure specific binding of Spo0A, Spo0A–P and Spo0ABD, the DNase I protection by the three activator forms at the spollG promoter was examined. A 410 bp DNA fragment from pUCIIGtrpA containing the spollG promoter was end-labelled at the BamHI site as described in the Experimental Procedures. This fragment was incubated with increasing amounts of Spo0A for 2 min and then treated with DNase I for 10 s as described in the Experimental Procedures. The resulting DNA fragments were separated on a denaturing gel and the gel was dried and exposed to x-ray film. Protection of the site 1 and 2 OA boxes was assessed by measuring the intensity of 32P-labelled bands within the protected regions and normalizing them to bands outside the protected regions (Figure 23).

In accordance with previous reports, Spo0A did not exhibit a high affinity for the OA boxes. Even at 800 nM, Spo0A displayed only 35% protection of site 1 and no protection of site 2 (Figure 23A). The results with Spo0A–P demonstrated that phosphorylation enhanced binding to the spollG promoter. Spo0A–P was able to completely protect both site 1 and site 2. Fifty percent maximum protection at sites 2 and 1 required 350 nM and 250 nM Spo0A–P, respectively (Figure 23B). Spo0ABD, like Spo0A, displayed 32% protection of site 1 and no protection of site 2 at 800 nM (Figure 23C). Thus, concentrations of Spo0ABD which markedly increased transcription from the spollG promoter (see Figure 15) did not result in stable binding. Two conclusions can be drawn from these data. First, enhanced DNA binding was not primarily responsible for the transcription stimulation properties of Spo0ABD and, by implication, stimulation of transcription by Spo0A–P does not solely depend on increasing the interaction of the C-terminus with the OA boxes at the promoter. This conclusion infers that since N-terminal inhibition of the C-terminus is not due to
hindrance of the HTH DNA binding motif it occurs by some other means. Secondly, the N-terminus of Spo0A does stabilize binding of the protein to DNA. This may be a consequence either of its mediating dimerization of Spo0A or its interaction with RNA polymerase.

2.0. The mechanism of transcription stimulation by activated Spo0A.

The previous experiments characterizing Spo0A, Spo0A-P and Spo0ABD demonstrated extensive similarity in the effect of the two activated forms of Spo0A on transcription stimulation from the spoIIIG promoter. In this section, the mechanism by which activated Spo0A stimulated transcription was explored.

2.1. Structural probing of open complex formation with potassium permanganate (KMnO₄).

Previous kinetic studies in our lab indicated that the initial binding of B. subtilis RNA polymerase to the spoIIIG promoter is independent of activated Spo0A (Bird et al., 1996). In Section 1, evidence supporting this conclusion was provided using EMSA. However, the complex formed by RNA polymerase initiates transcription at a very slow rate. Activated Spo0A accelerates the rate of initiation more than 50 times as measured by formation of complexes that are resistant to heparin (Bird et al., 1996). Thus, the structural characteristics of complexes generated by RNA polymerase with Spo0A-P or Spo0ABD should be similar to each other and should differ from those containing Spo0A. Likewise, complexes initiated with pApA and GTP or ATP and GTP should share some distinguishing feature to which the heparin-resistant state could be ascribed.
Figure 23. Activator binding at the spolIG promoter. The PvuI-BamHI fragment from pUC119GtrpA labelled at the BamHI site was incubated with RNA polymerase and increasing amounts of Spo0A (A), Spo0A-P (B) or Spo0ABD (C). Samples were treated with DNAsel and the fragments were separated by electrophoresis on an 8% polyacrylamide gel containing 7 M urea. Gels were dried and exposed to x-ray film. Protection of the site 1 (○) and 2 (●) OA boxes was determined by scanning the x-ray film and measuring the intensity of $^{32}$P-labelled bands within the protected regions and normalizing them to the intensity of bands outside the protected regions using a Molecular Dynamics Phospholmager SI and Imagequant software (v. 1.0).
Bird (1996) examined DNase I footprints at the *spolIG* promoter. While some features of the complexes could be distinguished, overall the patterns did not provide a mechanism for the stimulation of transcription by activated SpoOA. As another probe to more precisely define the stage of initiation affected by activated SpoOA, potassium permanganate (KMnO₄) assays were used to monitor DNA strand separation. KMnO₄ preferentially reacts with thymines on single strand DNA but not double strand DNA. The modified thymines can be selectively cleaved (Sasse-Dwight and Gralla, 1991) permitting identification of regions of strand separation. The structures of intermediate transcription initiation complexes were examined by incorporating different sets of initiating nucleotides into the transcription reactions. Ternary complexes containing the *PvuII* to *BamHI* DNA fragment from pUC11G*trpA* end-labelled at the *BamHI* site, Spo0ABD and RNA polymerase were formed in the presence of ATP, ApA, pApA plus GTP or ATP plus GTP. To track the process of promoter melting at *spolIG*, samples were treated with KMnO₄ and, following cleavage of the modified bases with piperidine (see Experimental Procedures), DNA fragments were separated on a denaturing polyacrylamide gel (Figure 24).

Neither RNA polymerase nor Spo0ABD alone induced sensitivity of the promoter to KMnO₄. In the absence of initiating nucleotides, the addition of Spo0A to RNA polymerase-promoter complexes induced very little sensitivity of the promoter to modification by KMnO₄, indicating that the majority of these complexes did not contain denatured DNA. The inclusion of ATP, pApA or pApA and GTP caused a slight increase in thymine reactivity in the -10 region of the promoter. The presence of ATP and GTP allowed modification of the DNA, with weak KMnO₄ sensitivity in the -13 to +13 region of the promoter on the non-coding strand. This low level of modification may represent an association of
the RNA polymerase with the promoter that was the source of the low level stimulation of transcription observed with Spo0A.

In contrast, the complexes containing Spo0A-P and RNA polymerase led to extensive denaturation of the promoter in the -3 to -13 region (on the non-coding strand) relative to the start site of transcription. The +1 site was not exposed despite the presence of ATP from the phosphorelay used to generate Spo0A-P and the fact that the first two bases in the transcript are adenines (Figure 8). When pApA and GTP were added, the denatured region was extended to the +2 position. The presence of ATP and GTP allowed the formation of a 26 bp denatured region from -13 to +13 on the template strand.

SpoOABD also stimulated the denaturation of DNA strands in RNA polymerase-promoter complexes without addition of nucleotides. Strong KMnO₄ reactivity was observed in the -3 to -13 region of the promoter. The addition of ATP or pApA did not alter the thymine reactivity pattern. Since complexes formed with SpoOABD did not contain any ATP, but did contain denatured DNA, this indicated that formation of the -3 to -13 denatured region was not dependent on the presence of ATP. Under ApA/GTP initiation conditions the region of denaturation was propagated downstream to the +2 position. At this point heparin-resistance was attained (Figure 14), thus, this state was dependent upon denaturation around the transcription start site and the initiation of RNA synthesis. In the presence of ATP and GTP, complexes contained DNA denatured from -13 to +13, like those formed with Spo0A-P.

Because denaturation of the +1 site would be required for initiation of RNA synthesis, the complex formed by Spo0A-P or Spo0ABD in which DNA between -3 to -13 was denatured does not represent a classical open complex, but rather an intermediate between closed and open complexes. Addition of ATP to the initiation reactions could lead to synthesis of the dinucleotide ApA,
yet the addition of ATP, or GTP which we tested as a control, did not lead to accumulation of complexes with the +1 site exposed. In contrast, the addition of pApA and GTP lead to exposure of the +1 and +2 sites on the template stand. The data in Figure 14 demonstrated that heparin-resistant initiated complexes could only accumulate when conditions allowed synthesis of at least a trimer, that is, in the presence of pApA and GTP. Thus, heparin-resistance coincides with exposure of the +1 site.

The non-reactivity of the thymines at +1 and +2 to KMnO₄ observed when Spo0ABD, RNA polymerase and ATP were incubated with the template was probably not due to protection of these residues by bound RNA polymerase by the following reasoning. Formation of an ApApG trimer led to exposure of the thymine at +2, which would be two bases 5’ to the RNA polymerization site, suggesting that bases at this site would be available to KMnO₄. Initiation with ATP would place the thymine at +1 in the same relative position, two bases 5’ to the polymerization site. Because the +1 thymine was not modified under ATP initiation, the conclusion was that it was not denatured.

The above experiments were repeated with the HindIII-BamHI DNA fragment end-labelled at the HindIII site to examine the denaturation pattern for the top (non-template) strand. None of the thymines on the top strand were exposed under ATP, or pApA plus GTP initiation conditions (Figure 25). Presumably, this lack of reactivity was due to the interaction of the coding strand with RNA polymerase (Simpson, 1979; Siebenlist et al., 1980; Brodolin et al., 1993; Buckle and Buc, 1994), preventing modification of T residues in this region. When complexes were formed in the presence of ATP plus GTP, positions -20 to -8 and the +17 position on the top strand were exposed.

Since Spo0A/RNA polymerase-promoter complexes contained only a low level of denatured bases upstream of +1 and could not initiate transcription
Figure 24. DNA strand separation at the *spollG* promoter induced by activated Spo0A. Transcription reactions containing 4 nM template DNA labelled at the *Bam*HI site with [$^{32}$P]ATP, RNA polymerase, 400 nM Spo0A, Spo0A~P or Spo0ABD and various initiating nucleotides (as indicated above each lane) were treated with KMnO$_4$ as described in the *Experimental Procedures*. Modified DNA was cleaved with piperidine and the cleavage products were analyzed on an 8% polyacrylamide gel containing 7 M urea. The gel was dried and exposed to x-ray film. Positions -13, +1 and + 13 are indicated as determined by Maxam and Gilbert sequencing of the same labelled fragment used in the KMnO$_4$ reactions run along side the KMnO$_4$ samples (not shown). A representation of the *spollG* promoter in pUC11GtrpA is shown on the left. Positions of the -10 sequence (□), and thymines sensitive to KMnO$_4$ modification are indicated by dots to the right (sensitive in the absence of ATP plus GTP) and dots to the left (additional sites sensitive in the presence of ATP and GTP).
Figure 25. KMnO₄ probing of the top (non-template) strand at the spollG promoter during the initiation of transcription. Transcription reactions containing 4 nM template DNA labelled at the HindIII site with [γ-³²P]ATP, RNA polymerase, 400 nM Spo0ABD and various initiating nucleotides (as indicated above each lane) were treated with KMnO₄ as described in the Experimental Procedures. Modified DNA was cleaved with piperidine and the cleavage products were analyzed on an 8% polyacrylamide gel containing 7 M urea. The gel was dried and exposed to x-ray film. Positions -20 and +17 are indicated as determined by Maxam and Gilbert sequencing of the same labelled fragment used in the KMnO₄ reactions run along side the KMnO₄ samples (not shown). A representation of the spollG promoter in pUC11GtrpA is shown on the left. Positions of the -10 sequence (□), and thymines sensitive to KMnO₄ modification in the presence of ATP and GTP are indicated by dots to the right.
efficiently, the results of KMnO₄ probing indicated that melting of the promoter upstream of +1 was critical for spollG stimulation. In contrast, both Spo0ABD and Spo0A−P with RNA polymerase induced denaturation of the spollG -10 promoter region, but not around the +1 site. The denatured region implies that activation of transcription from the spollG promoter requires the ability to induce this DNA change. Comparison of the denaturation pattern seen when Spo0A−P and Spo0ABD were added showed no significant differences, indicating that the general pathway of initiation stimulated by Spo0A−P and Spo0ABD was the same.

2.1.1. The pathway of promoter melting at spollG.

In the general model for transcription initiation, RNA polymerase associates with the template in a sequence-specific manner to form the closed complex. In order for RNA synthesis to proceed, hydrogen bonds between the DNA strands must be locally disrupted to allow incoming ribonucleoside triphosphates to hydrogen bond to the template strand. It is generally accepted that complexes formed at lower temperatures may represent transient intermediates in the initiation pathway at physiological temperature (Buckle and Buc, 1994). To determine the nucleotide positions at which DNA strand separation commenced within PspollG, transcription reactions containing the PvuII-BamHI fragment from pUC11GtrpA labelled at the BamHI end, were incubated with RNA polymerase, Spo0A−P and pApA and GTP at 25°C or 37°C. Samples were treated with KMnO₄, processed and the DNA fragments separated on a denaturing polyacrylamide gel as described in the Experimental Procedures. The x-ray film of the gel was scanned at 10 bits, gray scale on a AGFA Scan II scanner and the reactivity of each thymine was quantified on a
Figure 26. Effect of temperature on DNA denaturation induced by Spo0ABD. Transcription complexes containing RNA polymerase (40 nM), template DNA labelled at the BamHI site with $[\gamma^{32}]$ATP (4 nM) and Spo0AP (400 nM) were formed at 25°C (○) or 37°C (●) in the presence of ApA and GTP. Complexes were then probed with KMnO4 as described in the Experimental Procedures. Modified DNA was cleaved with piperidine and analyzed on an 8% polyacrylamide gel containing 7 M urea. The gel was exposed to x-ray film and the film was scanned and the reactivity (position indicated on x-axis) relative to that of the T at -13 was quantified using a Molecular Dynamics Phospholmager SI and Imagequant software (v. 1.0).
Phospholmager SI. The reactivity for each thymine position relative to the -13 site was determined and plotted in Figure 26.

At 37°C, each position from -13 to +2 was equally sensitive to modification by KMnO₄, as seen previously. At 25°C the pattern was markedly different. Relative to position -13, position -11 remained equally reactive, as it was at 37°C. Position -7 showed a 30% decrease in sensitivity to KMnO₄ and the thymines in region -4 to +2 were 65% less reactive. This indicated that the TA base steps at positions -13 and -11 of the TATA element were the most prone to melting during transcription initiation and the bubble expanded downstream to include -7, then -3 and eventually, in the presence of NTPs, +2.

2.2. Transcription by RNA polymerase from heteroduplex templates.

If creation of the partial denaturation was the critical function of activated Spo0A, then efficient transcription from PspollG heteroduplex templates containing an artificially denatured region equivalent to that generated in the presence of Spo0ABD should be independent of Spo0ABD. To test this hypothesis, heteroduplex DNA templates were created by annealing single strand DNAs produced from pBluescript clones carrying the spollG promoter. The spollG region was mutated to replace the template sequence of the spollG promoter with the coding strand sequence from position -7 to -14 or from -3 to -14 (see Experimental Procedures; Figure 10). By annealing single-strand DNA produced from wild type clones with single strands produced from mutants, it was possible to isolate DNA templates with denatured regions between -7 to -14 or -3 to -14 and which had either the template (T) or non-template (NT) sequence on both strands within the denatured region. Five templates (Table 4) were used in standard transcription assays and the sequences of the non-complementary regions are shown in Figure 11.
Three transcripts were observed from the heteroduplex templates after denaturing gel separation of the products of the transcription reactions. The RNAs were isolated and used as templates in primer extension experiments with the downstream IIG2 primer (Table 1) that had been end-labelled with \( \gamma^32P \)ATP and T4 polynucleotide kinase (see Experimental Procedures). The products of the extension reactions were separated on a denaturing polyacrylamide gel with products from dideoxy sequencing reactions using spollG as template and the same labelled primer. Primer extension analysis revealed that the major transcript produced from the WT, MB8NT and MB12NT templates was initiated from the same nucleotide position as observed in vivo for the spollG promoter (Figure 27). The start site for transcription from MB12T and MB8T was also the same as the in vivo start site, despite the lower level of consensus of the -10 region to a \( \sigma^A \) promoter (5' TATAtg 3', matches are in uppercase). Holoenzyme of E. coli has also been shown to initiate transcription from a specific site adjacent to the edge of a DNA bubble (Tripatara and deHaseth, 1993; Aiyar et al., 1994).

Two other transcription products smaller in size than the spollG wild type transcript were also generated from the MB12T and MB8T templates. Neither of these transcripts produced a primer extension product using the IIG2 primer (data not shown). The sizes of these RNAs were compatible with the hypothesis that they were derived from transcription that occurred in the opposite direction, using the wild type coding strand as the template. The focus of subsequent experiments was on the major transcript, comparing the effects of reaction conditions and of SpoOA on its synthesis.

The effect of the three forms of SpoOA on transcription from the heteroduplex templates was measured by quantifying the products after electrophoresis (Figure 28A). As expected, a low level of transcription was
Table 4. DNA templates used in transcription studies.

<table>
<thead>
<tr>
<th>Templates</th>
<th>Description</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>WTIIG</td>
<td><em>Pvu</em>I fragment of wild type <em>spolIG</em> promoter from pUCIIIG&lt;sub&gt;trpA&lt;/sub&gt;</td>
<td>Bird et al., 1993</td>
</tr>
<tr>
<td>MBWTIIG</td>
<td><em>Pvu</em>I fragment of wild type <em>spolIG</em> promoter from annealed pSKWTIIG+ and pSKWTIIG- single stranded DNAs</td>
<td>This study</td>
</tr>
<tr>
<td>MB8T</td>
<td><em>Pvu</em>I fragment harbouring an 8 nt heteroduplex containing the template sequence of the -10 region of the <em>spolIG</em> promoter on both strands</td>
<td>This study</td>
</tr>
<tr>
<td>MB8NT</td>
<td><em>Pvu</em>I fragment harbouring an 8 nt heteroduplex containing the non-template sequence of the -10 region of the <em>spolIG</em> promoter on both strands</td>
<td>This study</td>
</tr>
<tr>
<td>MB12T</td>
<td><em>Pvu</em>I fragment harbouring an 12 nt heteroduplex containing the template sequence of the -10 region of the <em>spolIG</em> promoter on both strands</td>
<td>This study</td>
</tr>
<tr>
<td>MB12NT</td>
<td><em>Pvu</em>I fragment harbouring an 12 nt heteroduplex containing the non-template sequence of the -10 region of the <em>spolIG</em> promoter on both strands</td>
<td>This study</td>
</tr>
</tbody>
</table>
seen from the reannealed wild type template in the absence of activated 
Spo0A. Transcription from the wild type template increased significantly in the 
presence of Spo0A-P, or Spo0ABD, but not Spo0A. RNA polymerase alone 
produced more RNA from each of the heteroduplex templates than from the wild 
type template. Transcription by RNA polymerase alone from the template 
containing the 12 nucleotide mismatch bubble with the non-template sequence 
in the bubble region (MB12NT) was equivalent to that from wild type templates 
in the presence of Spo0A−P. Reduction in the size of the bubble from 12 to 8 
nucleotides caused a 75% decrease in the amount of transcript produced 
(Figure 28B). Heteroduplex DNAs containing the template strand sequence of 
the promoter within the denatured region were not effective templates, 
regardless of the size of the denatured region. The presumption is that the 
lower level of transcription reflected the lower consensus in the -10 sequence. 
The observation that the degree of similarity to the -10 site affected the level of 
transcription from the denatured templates suggests that initiation was still 
dependent on the sigma subunit, which is an issue since in certain cases a 
DNA bubble obviates the need for sigma when the +1 site is exposed (Aiyar et 
al., 1994; Fredrick and Helmann, 1997). Slight increases in transcription by 
RNA polymerase alone were observed with the templates containing denatured 
regions, in agreement with the reports of Aiyar et al. (1994) that E. coli RNA 
polymerase will initiate transcription from heteroduplex templates lacking a 
sigma recognition sequence. The addition of Spo0A, Spo0A−P or Spo0ABD 
had no effect on the level of transcript produced from any of the heteroduplex 
templates (Figures 28, 29). The observation that the activated form of Spo0A 
did not stimulate transcription from the heteroduplex templates suggests that its 
effect on spoIIIG transcription was primarily in the formation of the denatured 
regions.
2.2.1. Temperature-dependence of transcription from heteroduplex templates.

It has been suggested that the major enthalpic barrier to transcription initiation may be related to denaturation of the DNA strands (reviewed in deHaseth and Helmann, 1995). To test whether this barrier was related to the denaturation induced by Spo0A\textsubscript{P}, the temperature dependence of transcription from the homoduplex and two of the heteroduplex templates was examined at temperatures ranging from 16°C to 42°C (Figure 29).

Standard transcription reactions containing RNA polymerase and the wild type, MB8NT or MB12NT heteroduplex template were assembled and assayed at the indicated temperatures. Without activator, transcription was not detected from the wild type template below 30°C and above this temperature, transcription increased gradually with temperature. With MB8NT as the template, transcription was detected at 16°C and the level of transcription increased linearly with temperature up to 30°C. In contrast, a high level of transcription from MB12NT was observed over the entire temperature range, with levels from 7 to 10-fold greater than from WT and 4-fold greater than from MB8NT. Therefore, the major enthalpic barrier to transcription from wild type templates below 30°C in the absence of activator was overcome by denaturation of the -3 to -14 region. Since Spo0A\textsubscript{P} induced an 11 bp denaturation analogous to that harboured by MB12NT (Fig. 24), this could account for the 8 to 10-fold increase in transcription from the wild type promoter in its presence. For both MB12NT and MB8NT, transcription at 16°C produced only 50% of the level of transcript observed at 37°C (Figure 29). This result suggested the existence of an additional temperature requirement that was not overcome by denaturation of the DNA strands between -3 and -13. This may reflect the need to extend the denatured region to beyond the +1 site.
Figure 27. Mapping of the Transcriptional Start Site from Heteroduplex Templates. RNA from \textit{in vitro} transcription reactions corresponding to the full length \textit{spolIG} transcript from the wild type or bubble templates (indicated above each lane) was isolated and used as template for primer extension reactions as described in the \textit{Experimental Procedures}. Products of the primer extension reactions and of a sequencing reaction using the same primer were analyzed on an 8% polyacrylamide gel containing 7 M urea. The nucleotide sequence of the region surrounding the transcription start site (\(+1\)) is shown on the left.
Figure 28. Transcription products from heteroduplex templates. A, wild type or heteroduplex template DNA (4 nM, indicated above each lane) was used in transcription reactions with Spo0A, Spo0A~P or Spo0ABD (400 nM). Reaction products were separated on a polyacrylamide gel as described in the Experimental Procedures and the gel was exposed to x-ray film. The region of the film recording the major transcript is shown. B, a graphic representation of the above results. The amount of transcript in each lane of the gel shown in A was quantified using a Molecular Dynamics PhosphorImager SI and Imagequant software (v. 1.0) and normalized to that produced from the wild type template in the presence of Spo0A~P (100%); RNAP alone (-----), with Spo0A (-----), Spo0A~P (-----) or Spo0ABD (-----).
Figure 29. Temperature dependence of transcription from heteroduplex templates. Reactions containing 4 nM template DNA (wild type (Δ), MB8NT (O) or MB12NT (●)), 80 mM potassium acetate, 40 nM RNA polymerase and the initiating nucleotides ATP and [α-32P]GTP in transcription buffer were incubated at the indicated temperatures. After 2 min complexes were challenged with a mixture of UTP, CTP and heparin and the incubations were continued at the same temperatures. Products of the reactions were separated on a polyacrylamide gel as described in the Experimental Procedures. The amount of transcript produced was quantitated using a Molecular Dynamics Phospholmager SI and Imagequant software (v. 1.0) and normalized to that produced from the MB12NT template at 37°C (100%).
3.0. Interaction of Spo0A with αCTD.

The C-terminus of the alpha (α) subunit of RNA polymerase (αCTD) participates in DNA binding, bending and transcription regulator contacts at different promoters (Russo and Silhavy, 1992; Ishihama, 1993; Ross et al., 1993; Mencia et al., 1996; Ptashne and Gann, 1997). Each component of αCTD action could directly influence transcription initiation since they can affect the spatial orientation of interacting proteins and DNA. While genetic evidence suggests that Spo0A interacts with the sigma subunit (Baldus et al., 1995; Schyns et al., 1997; Buckner and Moran, 1998; Buckner et al., 1998; Hatt and Youngman, 1998), other transcription regulators have been shown to interact with either the sigma or alpha subunits, or with both (reviewed in Busby and Ebright, 1997). Thus, evidence was sought for interaction of Spo0A and the polymerase alpha subunit at spolIG. The lab of Dr. M. Salas (Centro de Biologia Molecular (CSIC-UAM)) has perfected reconstitution of RNA polymerase from isolated subunits. In addition, Dr. Salas used RNA polymerases reconstituted with deletion mutants of alpha to study its interaction with the Bacillus subtilis bacteriophage activator protein p4. These reconstituted enzymes provide the reagents to test whether Spo0A interacted with the C-terminal region of alpha. Dr. Salas kindly provided enzymes containing deletions of the C-terminal 15 or 59 amino acids of the α subunit.

Equivalent amounts of the reconstituted polymerases were added separately to single round in vitro transcription reactions containing increasing amounts of Spo0ABD, and a template carrying the spolIG promoter. The products were separated by electrophoresis and quantified by analysis on a PhospholImager SI and Imagequant (v. 1.0) software (Figure 30). In each case, the polymerases containing deletion mutants of alpha were stimulated by Spo0ABD with about the same efficiency as was the polymerase reconstituted
Figure 30. Spo0ABD stimulation of transcription by RNA polymerase containing wild type or mutant alpha subunits. Reconstituted RNA polymerase was incubated with a DNA fragment containing the spollG promoter, ATP and $[\alpha^{-32}P]GTP$, and increasing amounts of Spo0A-P. After 2 min, a mixture of heparin, UTP and CTP was added to permit a single round of transcript elongation. Reaction products were separated by electrophoresis and full length transcripts were quantitated using a Molecular Dynamics Phospholmager and Imagequant (v. 1.0) software. Transcription is reported as fold stimulation over no Spo0A-P added for each polymerase preparation: △, wild type alpha; ●, alpha subunit truncated by 15 amino acids; ○, alpha subunit truncated by 59 amino acids.
with wild type alpha. Thus these results indicated that the C-terminus of the
alpha subunit was not required for RNA polymerase response to Spo0A-P. Of
note, however, was that the maximum level of transcription from PspollG by
RNA polymerase reconstituted with the mutant alpha subunits was only 30% of
that of wild type RNA polymerase (data not shown), suggesting that the alpha
mutations might affect the interaction of the polymerase with the DNA.

3.1. Effect of deletion mutants of the alpha subunit on the mechanism of
initiation.

Examination of mutants of other regulators have suggested that any
regulator may be capable of alternative pathways of transcription stimulation (Li
et al., 1994). As one test for whether the melting step was the same for the RNA
polymerase reconstituted with the three different alpha subunits, an
examination of the KMnO$_4$ modification pattern during initiation with Spo0ABD
and the mutant RNA polymerases was conducted.

The labelled $Pvu$-BamHI promoter fragment was incubated with the
reconstituted polymerases and Spo0ABD with various combinations of
nucleotides. KMnO$_4$ was added for 3 min and, after cleavage at modified
thymines, the fragments were resolved by electrophoresis (Figure 31A).
Complexes formed by all three forms of the polymerase plus Spo0ABD without
initiating nucleotides contained a denatured region which included the
thymines at -13 to -3. Addition of pApA and GTP led to further denaturation of
the +1 and +2 sites, and inclusion of ATP and GTP induced denaturation
between -13 and +13. The overall patterns of denaturation induced by all three
enzymes were similar to each other, in general agreement with the transcription
results, and to the pattern described earlier for wild type RNA polymerase
Thus, by this test, the three forms of polymerase generally used the same initiation mechanism.

Some differences were evident in the KMnO₄ reactivity pattern among the three polymerase forms. To clarify these differences, the x-ray film from Figure 31A was scanned and the reactivity of each thymine was quantified on a Phospholmager SI. The reactivity for each thymine position relative to the +1 site for the three polymerase forms under ATP plus GTP initiation conditions was determined and is shown in Figure 31B. Complexes containing wild type RNA polymerase displayed approximately equal degrees of sensitivity for all thymines between positions -13 and -3. Complexes formed with RNA polymerase lacking the C-terminal 15 amino acids of the alpha subunit displayed a similar pattern of thymine sensitivity as wild type RNA polymerase with the exception of the -3 and -4 positions, which were less reactive (1.4 and 2 fold, respectively). Complexes formed with RNA polymerase lacking the C-terminal 59 amino acids of the alpha subunit showed reduced thymine reactivity at positions -13 and -4 (1.8 and 4.5 fold, respectively) and enhanced reactivity at positions -11 and -7 (1.4 and 2.8, respectively). In section 2.2., the MB8NT template was transcribed much less efficiently than the MB12NT template indicating that lack of denaturation of the -3 and -4 positions decreased the transcription initiation rate. Since the polymerase with the alpha subunit lacking the C-terminal 59 amino acids did not denature these positions efficiently, the C-terminus of the alpha subunit may contribute to the denaturation step, and therefore initiation.
Figure 31. Denaturation of the spolIG promoter region induced by Spo0ABD plus RNA polymerase containing wild type or mutant alpha subunits. A, a P<sub>v</sub>uII-B<sub>a</sub>mpI DNA fragment of plasmid pUC11G<sub>trp</sub>A labelled at the BamHI site was incubated with 400 nM Spo0ABD, RNA polymerase reconstituted with wild type or mutant alpha subunits (40 nM) and combinations of nucleotides. After 2 minutes, the KMnO<sub>4</sub> was added and the samples processed to detect modified thymine residues. After cleavage with piperidine, the DNA fragments were separated by electrophoresis on denaturing polyacrylamide gels and detected by exposure to X ray film. The form of the alpha subunit (WT, wild type; -15, C-terminal 15 amino acids deleted; -59, C-terminal 59 amino acids deleted) used to reconstitute the polymerase (RNAP) is indicated above the panels and positions of the bases in the DNA, relative to the start site of transcription (+1) is indicated on the left. B, The sensitivity of individual thymine bases (x-axis) relative to the +1 site for each complex initiated with ATP and GTP in (A) was quantitated using a Molecular Dynamics Phospholmager SI and Imagequant software (V. 1.0). RNA polymerase containing wild type α (o); Δ15α (●); Δ59α (▲).
A

+ + + - - - - - - RNAP (WTα)
- - - + + + - - RNAP (-15α)
- - - - - - + + RNAP (-59α)
+ + + + + + + + Spo0ABD
- - + - - + - - ATP
- + + - + - + + GTP
- + - - + - + pApA

-13
+1
+13
3.2. EMSA detection of open complex formation by RNA polymerases containing deletion mutants of the α subunit.

Complexes containing the mutant polymerases, Spo0ABD and the spolIG promoter were also examined by electrophoretic mobility shift assays. The three polymerase forms and the end-labelled HindIII-BamHI fragment containing the spolIG promoter were incubated with or without either Spo0ABD or ATP plus GTP at 37°C for 3 min. Calf-thymus loading buffer was added and the samples were loaded onto a non-denaturing gel as described in the Experimental Procedures. (Figure 32). For the wild-type and Δ59 deletion, the presence of Spo0ABD stimulated the amount of complexes formed relative to the amount formed by RNA polymerase alone and including the initiating nucleotides ATP and GTP increased the level of complexes further. This was most dramatic for wild type RNA polymerase. The level of complexes formed in the presence of Spo0ABD and ATP/GTP were roughly proportional to the final levels of transcription which were detected in the experiment in Figure 30.

The complex formed by RNA polymerase reconstituted with wild type alpha, Spo0ABD and initiating nucleotides (ATP/GTP) had a detectably slower mobility than those formed by the polymerases containing the deleted alpha subunits. The change in mobility must reflect an altered conformation, most likely of the DNA within the complex. Thus, it appeared that polymerase reconstituted with the deleted alpha subunits interacted differently with the promoter than wild type polymerase. The data in Figure 23 also showed that compared to wild type polymerase, adding polymerase reconstituted with mutant alphas resulted in more complexes with the spolIG promoters, however the stability of these complexes was not addressed. As noted earlier, the level of transcription with the mutant polymerases was lower than for the wild type
Figure 32. Electrophoretic mobility shift assay of complexes formed by RNA polymerase containing mutant and wild type alpha subunits. The *PvuII-BamHI* DNA fragment of plasmid pUCII*GtrpA* labelled at the *BamHI* site was incubated with Spo0ABD (400 nM), RNA polymerase reconstituted with wild type (WTα) or mutant (-15α or -59α) alpha subunits (40 nM) and ATP plus GTP. After 3 min at 37°C, calf-thymus loading buffer was added and the samples were electrophoresed on a 5% non-denaturing polyacrylamide gel in TBE buffer (25). The gel was dried and exposed to X ray film. FD is free DNA.
polymerase. Thus, while the polymerases with the alpha deletions can still respond to Spo0A-P, they must have a lower rate of initiation.

4.0. Positive control mutations in the C-terminus of Spo0A.

Spo0A stimulates transcription from promoters transcribed by holoenzyme containing the major sigma factor, $\sigma^A$, or a secondary sigma factor, $\sigma^H$. Recently, regions within both sigma factors have been identified that are required specifically for response to Spo0A (Baldus et al., 1995; Schyns et al., 1997; Buckner and Moran, 1998). Conversely, a region within Spo0A involved in $\sigma^A$ specific stimulation (spanning residues 227-240) has also been identified (Buckner et al., 1998; Hatt and Youngman, 1998). A $\sigma^H$ specific interaction domain within Spo0A has not yet been defined, however, an alanine to valine substitution at position 257 has been demonstrated to abolish transcription stimulation of the $\sigma^H$ dependent spolIA promoter; a glutamic acid substitution at the same position also produced a sporulation deficient phenotype (Figure 9; Brehm et al., 1973; Ferrari et al., 1985; Kudoh et al., 1985; Perego et al., 1991). A257 lies within the C-terminal 15 residues of Spo0A, which if deleted, generates mutants blocked at stage 0 of sporulation (Ferrari et al., 1985). To investigate the role of this region of Spo0A in $\sigma^A$-dependent activation, two types of specific mutants of Spo0A were made. Two deletions of the C-terminal amino acids of Spo0A were created and valine scanning mutagenesis of the last 10 amino acids was carried out (Experimental Procedures).

The reasoning for this approach was as follows. If the alanine residue at position 257 (the 11th amino acid preceding the carboxy end of Spo0A) was the only amino acid of the C-terminal 15 required for its activation function, then deletion of the residues C-terminal to this position would still produce a sporulation proficient (spo$^+$) phenotype. If additional residues within this region
Table 5. Amino acid sequence of the C-terminus mutants of Spo0A.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amino Acid Sequence^a</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spo0AWT</td>
<td>EFIAMVADKLRLEHKAS</td>
<td>Ferrari et al., 1985</td>
</tr>
<tr>
<td>Spo0A9V</td>
<td>EFIAMVVDKLRLEHKAS</td>
<td>Ferrari et al., 1985</td>
</tr>
<tr>
<td>Spo0A153</td>
<td>EFIAMVEDKLRLEHKAS</td>
<td>Ferrari et al., 1985</td>
</tr>
<tr>
<td>Spo0AΔT</td>
<td>EFIAMVADKLRLEHKAS</td>
<td>This study</td>
</tr>
<tr>
<td>Spo0AΔ10</td>
<td>EFIAMVA</td>
<td>This study</td>
</tr>
<tr>
<td>Spo0AΔ15</td>
<td>EF</td>
<td>Ferrari et al., 1985</td>
</tr>
<tr>
<td>Spo0AV258</td>
<td>EFIAMVAVKLRLEHKAS</td>
<td>This study</td>
</tr>
<tr>
<td>Spo0AV259</td>
<td>EFIAMVADVLRLEHKAS</td>
<td>This study</td>
</tr>
<tr>
<td>Spo0AV260</td>
<td>EFIAMVADKVRLLEHKANH</td>
<td>This study</td>
</tr>
<tr>
<td>Spo0AV261</td>
<td>EFIAMVADKLVLEHKAS</td>
<td>This study</td>
</tr>
<tr>
<td>Spo0AV262</td>
<td>EFIAMVADKLRVEHKAS</td>
<td>This study</td>
</tr>
<tr>
<td>Spo0AV263</td>
<td>EFIAMVADKLRVHKAS</td>
<td>This study</td>
</tr>
<tr>
<td>Spo0AV264</td>
<td>EFIAMVADKLRLEYKAS</td>
<td>This study</td>
</tr>
<tr>
<td>Spo0AV265</td>
<td>EFIAMVADKLRLEHKAS</td>
<td>This study</td>
</tr>
<tr>
<td>Spo0AV266</td>
<td>EFIAMVADKLRLEHKVAS</td>
<td>This study</td>
</tr>
<tr>
<td>Spo0AV267</td>
<td>EFIAMVADKLRLEHKAV</td>
<td>This study</td>
</tr>
</tbody>
</table>

^aThe position at which the valine substitution occurred is underlined.
participated in transcription activation by Spo0A, then we expected this deletion mutant to produce a sporulation deficient (spo⁻) phenotype. If the latter case were true, then strains harbouring valine substitutions at critical positions within this region might also display a spo⁻ phenotype. Site-specific mutations were introduced into the spoOA gene by PCR amplification using the upstream HindIII10A primer and one of the mutagenic downstream primers listed in Table 1. To simplify the PCR approach, mutagenic primers were designed to anneal at the end of the coding sequence for the spoOA gene. Because of this, the transcription terminator was not included in the PCR product. A control in which the transcription terminator was removed from the wild type spoOA gene was included to ensure that deletion of the terminator did not effect production of Spo0A–P in vivo. Plasmid pKK0A, which harbours the full length wild type spoOA gene, was used as template (Figure 12). The mutated products were cloned into pGEM-T. The sequences of all spoOA alleles were verified. Plasmid DNA from each clone was cut at unique SphI and SstI sites and the corresponding spoOA gene fragments were cloned into pJM103 (Perego, 1993) digested with the same enzymes to create the pDRM series (Figure 13; Table 2). The amino acid sequence from position 251 to the end of each mutant generated is shown in Table 5.

pDRM plasmids harbouring the desired mutations (Table 2) were used to transform JH16304 to chloramphenicol resistance. Transformants resulting from Campbell-type recombination between the plasmid encoded spoOA gene and the chromosomal allele (Table 3) were selected on LB agar plates containing chloramphenicol and kanamycin. A homogeneous population of transformants was obtained and 10 representatives from each plate were chosen and examined for their ability to sporulate. Those displaying a spo⁻ phenotype were then tested for expression of the PspollG-lacZ fusion.
Table 6. The Effect of Spo0A Mutations on Spore Formation.

<table>
<thead>
<tr>
<th>Strain</th>
<th>cells/ml</th>
<th>spores/ml</th>
<th>frequency(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JH16304 (spo0A)</td>
<td>4.0x10^8</td>
<td>2.8x10^8</td>
<td>70</td>
</tr>
<tr>
<td>DR2001 (spo0A9V)</td>
<td>4.0x10^8</td>
<td>4.0x10^4</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>DR2002 (spo0A153)</td>
<td>2.0x10^8</td>
<td>&lt;1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>DR2003 (spo0A17)</td>
<td>3.0x10^8</td>
<td>3.0x10^8</td>
<td>100</td>
</tr>
<tr>
<td>DR2004 (spo0A10)</td>
<td>4.0x10^7</td>
<td>100</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>DR2005 (spo0A15)</td>
<td>2.2x10^7</td>
<td>&lt;1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>DR2006 (spo0A258)</td>
<td>4.0x10^8</td>
<td>&lt;1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>DR2007 (spo0A259)</td>
<td>4.0x10^8</td>
<td>3.0x10^8</td>
<td>75</td>
</tr>
<tr>
<td>DR2008 (spo0A260)</td>
<td>2.0x10^7</td>
<td>500</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>DR2009 (spo0A261)</td>
<td>4.0x10^8</td>
<td>4.0x10^8</td>
<td>100</td>
</tr>
<tr>
<td>DR2010 (spo0A262)</td>
<td>5.0x10^8</td>
<td>3.0x10^8</td>
<td>60</td>
</tr>
<tr>
<td>DR2011 (spo0A263)</td>
<td>5.0x10^8</td>
<td>4.0x10^8</td>
<td>80</td>
</tr>
<tr>
<td>DR2012 (spo0A264)</td>
<td>3.7x10^8</td>
<td>3.0x10^8</td>
<td>81</td>
</tr>
<tr>
<td>DR2013 (spo0A265)</td>
<td>5.0x10^8</td>
<td>3.0x10^8</td>
<td>60</td>
</tr>
<tr>
<td>DR2014 (spo0A266)</td>
<td>3.8x10^8</td>
<td>2.7x10^8</td>
<td>71</td>
</tr>
<tr>
<td>DR2015 (spo0A267)</td>
<td>5.0x10^8</td>
<td>1.5x10^8</td>
<td>30</td>
</tr>
</tbody>
</table>

\(^a\)Cells were grown in SSM with Kan and Cm and sporulation frequency determined as described in the Experimental Procedures.
Figure 33. The effects of *spo0A* Mutations on expression of a *spolIG-lacZ* transcriptional fusion. The indicated strains (Table 3) were cultured as described in the Experimental Procedures. Samples were collected at 1 hr intervals from mid-log (T₂) to well into stationary phase (T₁ to T₄) and assayed for β-galactosidase activity (Ferrari *et al.*, 1986). T₀ denotes the end of exponential growth.
Of the 11 mutant strains created (Table 3), DR2004, DR2006 and DR2008, which carry the *spoOAΔ10, spoOAD258V* and *spoOAL260V* alleles, respectively, had a sporulation frequency of <0.1% (Table 6). These three mutant strains, in addition to three previously reported C-terminal mutations that resulted in a spo− phenotype, DR2001, DR2002 and DR2005, which carry the *spoOA9V, spoOA153* and *spoOAΔ15* alleles, respectively, were the focus of further study. These six strains were analyzed for expression from the *spollG-lacZ* promoter fusion (Figure 33). The strain carrying a wild type SpoOA gene showed stimulation of the *spollG* promoter beginning at T1 and reaching a maximum at T3. Deletion of the C-terminal 10 and 15 amino acids (DR2004 and DR2005) resulted in a reduction of *spollG* promoter activity to 14% and 10% that of wild type. This indicated that residues within this segment of SpoOA other than A257 might be involved in stimulation of the *spollG* promoter. The valine scanning mutant strains DR2001, DR2006 and DR2008 as well as the A257E mutant strain DR2002, showed decreased expression of the promoter fusion to less than 10% that of wild type, similar to a *spoOA* null strain (Buckner *et al.*, 1998; Hatt and Youngman, 1998).

To be sure I was examining positive control mutants of SpoOA, I tested its repressor function at the *abrB* promoter. The product of the *abrB* gene, AbrB, is a repressor that regulates transcription from several promoters (Perego *et al.*, 1988; Strauch *et al.*, 1990). One of these promoters encodes an extracellular protease. The *abrB* promoter is repressed by SpoOAP to allow protease production in the search for alternative energy sources before sporulation is triggered (Perego and Hoch, 1988). The ability of the SpoOA mutant proteins to bind and repress transcription was monitored by qualitative assay for protease activity on milk agar plates. Each of the mutant strains displayed normal levels of protease activity compared to wild type (Table 6). These results indicated
that amino acids within the C-terminal 15 residues, specifically A257, D258 and L260, were necessary for the activation function of Spo0A at the spollG promoter. Most notably, the inability of the spo0A9V mutant to stimulate spollG-lacZ expression suggested that this region of Spo0A was required for activation of both $\sigma^A$ and $\sigma^H$-dependent promoters.
DISCUSSION

Changes in the length of the *E. coli* σ^70 promoter spacer region decrease the efficiency of transcription initiation (Helmann and Chamberlin, 1988; Gross *et al.*, 1992; deHaseth and Helmann, 1995). This decrease in efficiency led to the hypothesis that the sigma factor binds to both -35 and -10 sequences to stimulate the DNA strand denaturation required for isomerization of closed to open complexes (Warne and deHaseth, 1993; Dombroski *et al.*, 1996). Kenney *et al.* (1989) hypothesized that the length of the *spollG* spacer region (22 bp instead of the optimum 17 bp) prevents effective contact of the sigma subunit with the -35 and -10 sequences, explaining why transcription is low in the absence of Spo0A~P. This investigation has revealed two principal focal points for activated Spo0A in transcription initiation from the *spollG* promoter. First, structural probing of activator-RNA polymerase-*spollG* complexes demonstrated that phosphorylation generated a form of Spo0A that interacted with RNA polymerase to stimulate melting of the *spollG* promoter. Non-phosphorylated Spo0A did not stimulate this step. Since RNA polymerase binds rapidly to the *spollG* promoter, the observation that the polymerase alone cannot create this denatured region identified the barrier to initiation. Second, Spo0A~P stabilizes the uninitiated ternary complex and the addition of initiating nucleotides shuttles these unstable open complexes into stable initiated complexes. This mechanism groups Spo0A in a class of activator (which includes the regulators NtrC (North and Kustu, 1997), NifA (Buck and Cannon, 1989) DctD (Huala *et al.*, 1992) and N4SSB (Miller *et al.*, 1997)) that functions not through recruitment of RNA polymerase to the promoter, but through modification of pre-bound RNA polymerase complexes.
1.0. The pathway of open complex formation at *spolIG*.

The mechanics of promoter regulation by SpoOA has been most extensively studied at the *spolIG* promoter. Previous kinetic and structural analysis suggested a mechanism for SpoOA activation of transcription (eq. 2)

\[
R + P \rightarrow C_I \quad \text{SpoOA} \rightarrow C_{II} \quad \text{NTPs} \rightarrow \text{RP}_{ITC}
\]

in which RNA polymerase (R) was able to rapidly form an unstable complex with promoter DNA (P), designated C_I (Bird, 1996; Spiegelman et al., 1995). However, this binary complex was inefficient at initiating transcription (Bird et al., 1993; Bird et al., 1996) and the -35 region but not the -10 region of the *spolIG* promoter was fully protected from DNase I presumably due to the lengthy spacer region. Addition of SpoOA led to formation of the C_{II} complex, characterized by helix distortion (as evidenced by the appearance of DNase I hypersensitive sites) and more extensive protection of the -10 region. This process was hypothesized to involve interaction of SpoOA and RNA polymerase since the binding sites for these two proteins overlap. However, this complex was still inefficient at initiating transcription. The presence of SpoOA~P caused rapid conversion of initially formed C_I complexes to transcription ready C_{III} complexes, characterized by complete protection of the promoter region. The suggested role for SpoOA~P was to aid RNA polymerase in contacting the -10 and -35 regions simultaneously by a cooperative distortion of the DNA helix, generating a complex amenable for transcription. The studies of Bird et al. (1996) demonstrated the presence of multiple steps in the initiation pathway at
spollG but they did not determine which step(s) in the transcription reaction path were specifically affected by Spo0A~P.

1.1. Establishment of a denatured region upstream of the transcription start site.

The results of this study have extended our understanding of steps in initiation that are influenced by Spo0A~P and are summarized in the reaction scheme below,

\[
\begin{align*}
1 & \quad 2 \quad 3 \\
\text{NTPs} & \\
R + P & \leftrightarrow \text{RPc} \leftrightarrow \text{ARP}_n \leftrightarrow \text{APR}_{\text{ITC}} \rightarrow \text{RP}_{\text{IEC}} \\
(3\text{-11 nts}) & \\
\end{align*}
\]

where R is the RNA polymerase, P is the spollG promoter and A represents Spo0A~P. The results shown in Figures 16 and 17 verified previous reports that RNA polymerase was capable of forming a closed complex (RPc) with the spollG promoter in the absence of Spo0A~P (stage 1; termed the Ci complex by Bird et al., 1996). The DNA distortion that arose upon addition of Spo0A to the Ci complex reported by Bird et al. (1996), to form the C\text{n} complex, was probably not due to DNA denaturation since the SpoOA-RNA polymerase complex was not reactive to KMnO\text{₄} (Figure 24). This explains the poor capacity of Spo0A to stimulate transcription.

In contrast, ternary complexes formed with Spo0A~P, the C\text{III} complex of Bird et al. and the APR\text{n} complex in the reaction scheme above, no longer exhibited the DNase I hypersensitive sites of the C\text{II} complex but contained DNA fully denatured in the -10 region of the promoter. To create this denatured region, strand separation commenced specifically at the -13 and -11 positions on the template strand (Figure 26). These thymine bases are located within the
TATA element and were reported to be the most likely to melt (see deHaseth and Helmann, 1995 for review). The denaturation extended downstream to include the -7 position and stopped at the -3 position. The requirement of Spo0A-P for transcription by RNA polymerase from spollG could be completely bypassed by a heteroduplex template harbouring denatured DNA from positions -13 to -3 (Figure 28A, B). Thus, Spo0A-P functions by accelerating the formation of a specific intermediate in the RPc to RP_{ITC} (initial transcribing complex) transition, in which the DNA is denatured upstream of the transcription start site. The 'bubble' templates supported maximal rates of initiation because these templates contained the wild type -35 sequence and an exposed -10 region, allowing polymerase to bind rapidly and initiate readily. Transcription from the bubble template remained dependent on the sigma subunit since templates which did not contain the $\sigma^A$ -10 recognition sequence were poor templates for holoenzyme. Other heteroduplex templates would be required to analyze precisely the positional requirements for the denatured region in open complex formation. The experiments reported here do not resolve whether the C_{II} complex of Bird et al. (1996) is a legitimate intermediate in the pathway of promoter melting or a non-productive association of the non-phosphorylated regulator with the polymerase and DNA.

1.2. The initiation of RNA synthesis.

Addition of the NTPs pApA and GTP at stage 2 of equation 3 leads to the formation of an initial transcribing complex, RP_{ITC}. The studies of Bird et al. (1996) did not investigate the pApA plus GTP initiation and so did not examine the structural characteristics of these initiated complexes. This complex was characterized by exposure of the +1 site (Figure 24) and resistance to heparin (Figure 14), the hallmark of stable open complex formation. Polymerization of
the first three nucleotides was necessary to establish a classical, stable open complex. At any point prior to this, RNA polymerase could be displaced by heparin.

Goldfarb and co-workers have studied transcription at two unstable *E. coli* promoters, λP_L and the P1 promoter of the ribosomal RNA operon *rrnB* (Borukhov *et al.*, 1993; Severinov and Goldfarb, 1994). A key observation of these promoters was the requirement for initiating nucleotides for the formation of an RNA polymerase-promoter complex that was stable to heparin challenge. The stabilizing effect of nucleotide polymerization was proposed to be due to entrance of the 5' end of the of the growing RNA chain into a tight-binding-site (TBS) on RNA polymerase which triggers the enzyme to lock on the DNA. The minimum length of the nascent transcript required for full stability of the transcription complex was four nucleotides. At the *spolIG* promoter, conditions which permitted synthesis of a 3-mer transcript allowed formation of heparin stable complexes, although only to 40-50% the level observed when complexes synthesized an 11-mer transcript (Figure 14). By analogy with the results of Goldfarb *et al.* (1994), synthesis of a 4-mer transcript would be expected to produce maximum levels of heparin-resistant complexes if σ^A^-holoenzyme also harbours a TBS.

The requirement for nucleotide polymerization for formation of a stable complex is found at other *Bacillus* promoters (Dobinson and Spiegelman, 1987; Whipple and Sonenshein, 1992; Rojo *et al.*, 1993; Juang and Helmann, 1994a) and some *E. coli* promoters (Ohlsen and Gralla, 1992; Wang and Gralla, 1996) and probably reflects the fact that intermediates between closed and open complexes are in rapid equilibrium. This rapid equilibrium would allow the initial complex to isomerize to one which can initiate efficiently. Promoter
escape into an initial elongating complex (RP_{IEC}) by the transcription complex occurs in stage 3 of equation 3.

The ARP_n complex in equation 2 was distinctly different from the classical open complex which is defined by i) exceptional stability and resistance to competitive inhibition and ii) denaturation around the +1 site, the start site for transcription initiation. The APR_n complex was heparin-sensitive and the initial denatured region did not include +1 (Figure 24). The APR_n complex adopted the characteristics of a classical open complex when conditions permitted the synthesis of a 3-mer nascent transcript. This, however, does not directly address whether RNA synthesis is absolutely required for stabilization or whether denaturation of the +1 site triggers this state. The synthesis of a heteroduplex template that includes the +1 position within the single-strand region could be used to separate the contributions of the two processes.

1.3. Start site selection in the initiation of transcription.

Primer extension analysis of transcripts isolated from the MB12NT bubble template (Figure 27) revealed multiple minor bands in addition to the major band. These minor bands could arise in two possible ways. Goldfarb and co-workers have identified the process of transcript slippage at two E. coli promoters (Borukhov et al., 1993; Severinov and Goldfarb, 1994). This process relies on nucleotides from -3 to +3 being of only one or two different types and repetition of the template sequence in the vicinity of the start site. This allows RNA polymerase to synthesize a short oligonucleotide, then slip back and repeat the synthesis without releasing the transcript. A repetitive AAG sequence is found near the transcription start site of the spollG promoter (AAGCA_{+1}AG). However, the C at position -1, prevents slippage from occurring,
so this process does not explain the extra bands observed in the primer extension experiments.

An interesting structural characteristic of \textit{PspollG} may provide an explanation as to the source of these additional primer extension products. In addition to its unusual spacer length, the \textit{spollG} promoter is exceptional in the position of the -10 element in relation to the +1 site. At consensus promoters, the -10 position corresponds to the second T of the element (TATAAT, underlined) and transcription is almost always initiated 10 nucleotides downstream of this point. At \textit{spollG}, the -10 region is displaced 2 bases 5' to the +1 site (TATACT, the -10 position is underlined), placing the expected transcription start site between positions -4 and -2. Since formation of the trimer AAG produces a stable open complex and the sequence is found at two places near the start site of transcription, from -4 to -1 and +1 to +3, it is possible that RNA polymerase might initiate transcription from the A at -4 in addition to the normal +1 position. Both would generate transcription complexes bearing a nascent transcript that could be extended to full length products with some being up to 4 nucleotides longer than the transcript started from +1. The detection of a single primer extension product from the wild type promoter in the presence of Spo0A~P suggests that the regulator restricts the start site selection point by sigma to the +1 site. However, in the denatured template transcriptions Spo0A~P was not added, possibly loosening the regulation of start site selection.

1.4. Open complex formation follows a melting-from-the-edge pattern.

Three models have been proposed to describe the process of DNA melting at promoters (deHaseth and Helmann, 1995). One model suggests that DNA melting occurs simultaneously over a 12 bp or more region in what has
been termed a concerted reaction. A second model suggests that melting occurs in discrete steps involving several stable intermediates in what is termed a stepwise reaction. Based on studies at the λP_R promoter, a third model has been suggested in which melting initiates between -10 and +1 and propagates outwards in both directions during formation of the open complex (deHaseth and Hellman, 1995).

In this investigation, a striking feature of the denaturation of the spollG promoter was that it proceeded by way of a stable intermediate: positions -13 to -3 were denatured first (commencing in the TA base steps of the TATA element) without initiating nucleotides and then the denaturation extended beyond the +1 site in the presence of nucleotides (Figure 24). This stepwise denaturation conformed to a 'melting from the edge' pattern, since denaturation of the +1 site began upstream of that position. This pattern has been reported for other promoters, for example the trnS promoter of B. subtilis, which also showed DNA denaturation initiating within the -10 region of the promoter and its unidirectional movement toward +1 (Juang and Helmann, 1995). The formation of a melted intermediate which stops short of the +1 site is reminiscent of the open complex formed by E. coli σ^{70}-holoenzyme in the absence of Mg^{2+}, that contains DNA denatured from the -10 region to the -1 position (Suh et al., 1993). It is viewed as an intermediate in the formation of a functional open complex since the addition of Mg^{2+} extends the bubble to +2. The interpretation of the melting pattern at spollG is that the two-step denaturation is a feature of this promoter and not a necessary component of regulation by Spo0A~P.
2.0. How Spo0A--P might stimulate transcription.

How Spo0A--P allows sigma to recognize the -10 region at spoIIIG is not clear. Several means to achieve the same end, stimulation of transcription, are discussed below.

2.1. Repositioning of the σ subunit.

The importance of the -35 recognition region of σ70 was investigated by Kumar et al., (1994) at transcription factor-dependent and independent promoters. Reconstituted holoenzymes containing sigma subunits with deletions in region 4.2 were unable to transcribe factor-independent promoters but could transcribe promoters activated by Class II transcription factors. Thus, the sigma/-35 contacts were not essential for stimulation by Class II activators. It was proposed that these contacts were replaced by protein-protein contacts between RNA polymerase and the activator. At spoIIIG, Spo0A is a Class II activator since it binds a region of DNA that overlaps the -35 site and contacts the sigma subunit (Satola et al., 1992; Bird et al., 1993; Baldus et al., 1995; Schyns et al., 1997; Buckner and Moran, 1998; Hatt and Youngman, 1998).

The simplest model for how Spo0A--P stimulates DNA melting is one in which Spo0A--P, by contacting both the DNA and the sigma subunit, allows sigma to release its initial -35 DNA contacts and replaces them with Spo0A--P-RNA polymerase contacts. The sigma subunit then repositions to contact the -10 sequence leading to DNA denaturation and an increase in transcription. The positioning of the 0A boxes at spoIIIG (overlapping the -35 sequence of the spoIIIG promoter) and the Spo0A--P induced denaturation of the -3 to -13 region shown in this work are compatible with this scheme.

Repositioning of a polymerase subunit has been previously reported (Belyaeva et al., 1996). Activation of Class II promoters by CAP is a two step
process involving two regions of the CAP protein: AR1 mediated 'anti-inhibition' of the αCTD and AR2 mediated interaction with the N-terminus of alpha. The binding of CAP to the DNA immediately upstream of the -35 element precludes the αCTD from interacting at its preferred location within the same DNA region. Consequently, the αCTD is repositioned 5' at a less preferred location (Belyaeva et al., 1996) but productive contacts between the CAP and the polymerase stimulate initiation from the promoter.

2.2. A conformational change in the RNA polymerase σ subunit.

The universal rate limiting step at all σ70-dependent promoters studied to date is proposed to relate to the isomerization of the initial closed complex to a second complex in which RNA polymerase has undergone a conformational change but has not yet opened the DNA helix (Hawley and McClure, 1982; Roe et al., 1984; Roe et al., 1985; Leirmo and Record, 1990). This conformational change coincides with enhanced protection of the -10 region and nucleotides downstream of +1 (Leirmo and Record, 1990). This implies that regulators that stimulate transcription at non-consensus promoters through contacts with σ70 modify the RNA polymerase in some manner. Dobromski et al. (1996), proposed that transcription regulators could induce a conformational change in sigma by altering the interaction of regions 2.4 and 4.2 of the sigma subunit. These regions contact the -10 and -35 consensus sequences and their normal spacing establishes an optimum -10 to -35 distance. Modification of the 2.4 and 4.2 region interaction could allow the sigma subunit to bind promoters with unusual spacing.

A model in which Spo0A induces sigma to contact both -35 and -10 sequences implies that Spo0A and sigma bind to the DNA simultaneously. At the spolIIG promoter there are two 0A boxes (see Figure 8), one of which
overlaps the -35 consensus sequence completely. The hydroxyl-radical, DNAse I and methylation protection footprints of Spo0A-P binding show that it binds across the DNA minor groove 5' to the 0A box on one face of the helix (Greene and Spiegelman, 1996) and within the 0A boxes (Strauch et al., 1990). Thus it is possible that Spo0A and sigma could contact the 0A box/-35 sequence simultaneously, and so the sigma modification model cannot rule out. However, this model is not favored for activation of *spolIG* because it would require a large lateral displacement of regions 2.4 and 4.2 of sigma to contact both the -10 and -35 sequences at the same time. It seems simpler to assume that only one protein binds to the -35 region at a time.

An *in vitro* system in which σ^A or σ^H is bound at a promoter DNA in the absence and presence of Spo0A-P might allow the alternative mechanisms of sigma repositioning or modification to be resolved. Such an experiment is feasible, since N-terminal deletions and polypeptides of σ^70 have been reported to bind specifically to double and single-strand promoter DNA on their own (Dombroski et al., 1992; Dombroski et al., 1993; Dombroski et al., 1996; Dombroski, 1997) and these characteristics can be exploited by using heteroduplex templates. However, the analysis may be complicated by requirements for additional contacts between sigma and core polymerase.

One model for Spo0A activation of the *spolIG* promoter would assume that the sigma subunit is relatively rigid and that activated Spo0A acts to lever it into position where it contacts the -10 sequence. Other models for the activity of Spo0A-P are possible. For example, the MerR regulator binds to the *mer* operon promoter inducing a DNA deformation as evidenced by DNAse I hypersensitive sites (Parkhill et al., 1993). The deformation may allow the sigma subunit to bridge the 19 bp distance between the -10 and -35 sequences at this promoter. This model seems unlikely for Spo0A because it does not
induce dramatic DNA changes in the promoter as measured by DNAsel I (Bird et al., 1996; Greene and Spiegelman, 1996), hydroxyl radical (Greene and Spiegelman, 1996), or KMnO₄ (Figure 24) experiments.

3.0. The Spo0A-P paradigm.

The current model for Spo0A activation of the spoIIG promoter is summarized in Figure 34. Since a spoIIG-RNA polymerase complex can be demonstrated by electrophoretic mobility shift assays (Figures 16 and 17; Cervin et al., 1998) and kinetic assays show that Spo0A-P has no effect on the binding of the polymerase to the promoter (Bird et al., 1996), the first stage of the reaction is the binding of σ⁰ holoenzyme to the promoter without Spo0A. In DNAsel I protections assays RNA polymerase bound to the -35 region of the spoIIG promoter, and the -10 region was only slightly protected (Bird et al., 1996). The -35 region is also involved in the initial binding of σ⁰⁰ E. coli RNA polymerase (Gross et al., 1992; deHaseth et al., 1998). This suggests that the barrier preventing transcription from the spoIIG promoter may be similar to the barrier to transcription initiation at σ⁰⁰-dependent promoters, protection of the +1 region of the promoter by RNA polymerase.

The second stage of the initiation reaction is the binding of activated Spo0A to the RNA polymerase-spoIIG complexes. Since the 0A boxes overlap the -35 sequence of the spoIIG promoter, by contacting both the DNA and the sigma subunit, activated Spo0A allows sigma to release the -35 contacts without dissociation of the RNA polymerase from the DNA. The sigma subunit then repositions to contact the -10 sequence which results in protection of the -10 region, denaturation of the -3 to -13 region, and an increase in the rate of initiation. The Spo0A-P induced denaturation of the -3 to -13 region shown in this work is compatible with this scheme.
Figure 34. The model for Spo0A-P stimulation of the spollG promoter. (A) At the spollG promoter (double lines), the -10 and -35 RNA polymerase consensus sequences (black boxes) are separated by 22 bp instead of the optimal 17 to 18 bp. The core RNA polymerase is shown as a large oval. Regions 4.2 and 2.4 of the sigma subunit, which contact the -35 and -10 sites respectively, are represented as polygon structures connected by a linker. For simplicity, only the RNA polymerase consensus sequences and site 2 0A boxes (striped boxes) are shown. (B) The initial interaction is postulated to occur between the -35 sequence of the promoter and region 4.2 of σ^A. (C) The two domain structure of Spo0A-P is represented as a small gray oval (N-terminus) and a white oval (C-terminus). The postulated RNA polymerase interaction domain is depicted as a projection on the N-terminus. Binding of Spo0A-P to the RNA polymerase-promoter complex causes release of the σ^A-35 contacts. σ^A contacts the -10 region resulting in denaturation of the DNA strands upstream of +1, shown as a bubble. (D) When ATP and GTP are present a nascent RNA chain (indicated by the thick line) is synthesized and the denatured region is extended downstream.
In the third stage of the reaction, addition of ATP and GTP or pApA and GTP to the complex with the -13 to -3 denaturation leads to extension of the denatured region past +1 due to polymerization of the nascent transcript. The temperature dependence of transcription above 25 °C from wild type templates in the presence of Spo0A-P was similar to the dependence of transcription from the 12 bp bubble template in the absence of Spo0A-P over the same temperature range (i.e. an increase of 15% in the amount of transcription in going from 25°C to 37°C; Figures 19 and 30). This suggests that Spo0A-P did not facilitate the second denaturation step of DNA bases between -3 and +13 to yield initial transcribing complexes.

3.1. Comparison of activation by Spo0A to activation by other transcription activators.

Despite the large number of activating response regulators that have been identified, there is very little detailed information about how they stimulate transcription initiation. Fundamental to the mechanism proposed for Spo0A are the following: i) the sigma factor used is intrinsically active; ii) the RNA polymerase specifically binds the promoter but cannot melt the DNA strands; iii) activator binding near the -35 region stimulates conversion of closed to open complexes. The *E. coli* regulator NtrC has been found to activate transcription by modifying the activity of σ^{54}-holoenzyme (see Introduction). Like Spo0A, the C-terminus of the α subunit of RNA polymerase is not essential for transcriptional activation of σ^{54} holoenzyme by NtrC (Lee et al., 1993). Although σ^{54}-holoenzyme is incapable of catalyzing DNA denaturation on its own at any promoter *in vivo* (see Introduction), NtrC-P stimulates transcription by inducing open complex formation by σ^{54}-holoenzyme and stabilizing the open complex in a manner similar to Spo0A-P. The overall mechanism of activation by λcl
and CAP AR3 are also quite similar to that found for Spo0A-P. These transcription factors activate transcription by stimulating conversion of closed to open complexes through interaction with the sigma subunit (Busby and Ebright, 1997; Li et al., 1997 and the references therein). Since the genetic evidence suggests that λcl (Bell et al., 1990b; Williams et al., 1991; West et al., 1993; Kuldell and Hochschild, 1994; Li et al., 1994; Niu et al., 1996) interacts with region 4 of sigma, as does Spo0A-P (Baldus et al., 1995), similar protein-protein interactions may govern these regulation events.

Denaturation of the DNA strands is likely to be a common target for positively activating response regulators, suggesting that the general mechanism will be to promote interaction of region 2 of the sigma subunit with the -10 sequences. For cases such as the spoIIIG promoter, where the initial RNA polymerase binding doesn't involve -10/sigma interaction, the response regulator might displace the sigma subunit from the initial contacts. At other promoters where the RNA polymerase does not make a closed complex on its own, the response regulator may act to stabilize an intermediate state of binding, increasing the probability of effective contact between -10 and sigma.

3.2. Transcription from non-consensus promoters.

The results regarding the mechanism of RNA polymerase stimulation by Spo0A-P are of interest because it could provide a model for understanding stimulation from other promoters to which RNA polymerase binds but cannot initiate because of deviations in spacer length or ineffective -35 consensus sequences. Helmann (1995) compiled and analyzed 263 (confirmed and putative) σA-dependent promoters from *B. subtilis*. Of these, 8 did not have a discernible -35 element and 30 had a spacer distance of less than 17 bp; most of these promoters harbour a highly conserved TG dinucleotide at positions -15
and -14, similar to the extended -10 promoters of *E. coli* (Bown *et al.*, 1997). Transcription can be initiated from these promoters because the TG dinucleotide relieves the requirement for contacts with the -35 hexamer by creating an alternative contact point for sigma (Ponnambalam *et al.*, 1986; Kielty and Rosenberg, 1987; Chan *et al.*, 1990; Kumar *et al.*, 1993; Burns *et al.*, 1996). The position of the 0A boxes may allow Spo0A-P to provide the same service at the *spolIG* promoter. Of the 27 promoters with a spacer length greater than 18 bp, most do not contain the TG dinucleotide. Because insertion of a single base pair can drastically impair promoter activity (Stefano and Gralla, 1982; Mulligan *et al.*, 1985; Ayers *et al.*, 1989; Warne and deHaseth, 1993), expression from these promoters is likely activator-dependent. Other two-component regulators or unrelated activators that interact with σ^A_ may act in a similar manner to Spo0A to activate expression of promoters with deviant spacer lengths or those lacking a -35 element.

4.0. The role of the α subunit of RNA polymerase in transcription.

Many positive regulators have been shown to stimulate transcription through contacts with the α subunit of RNA polymerase (Ishihama, 1993). In addition, a single activator can have multiple targets in the transcriptional machinery (Busby and Ebright, 1997). An activator contact site (Contact site I) has been identified within the carboxy-terminal 73 amino acids of the alpha subunit of *E. coli* RNA polymerase (Figure 2; reviewed in Ishihama, 1993) and deletion of the analogous region from the *B. subtilis* alpha subunit (C-terminal 59 amino acids) destroys polymerase response to the bacteriophage φ29 transcription activator p4 (Mencia *et al.*, 1996; Mencia *et al.*, 1998). Positive control mutations near the carboxy-terminus of p4 and Spo0A exhibit similar phenotypes (Figure 35). In particular, Rojo and co-workers have
shown that deletion of the 10 terminal amino acids of gp4 eliminates in vitro transcription stimulation and the single point mutations, R120Q and L117A, abolish the protein's ability to stimulate transcription by RNA polymerase without affecting its DNA binding activity (Rojo et al., 1990; Mencia et al., 1993). These residues are critical for stimulatory function and it has been suggested that they form part of an activating domain that makes direct contact with the α subunit of RNA polymerase to activate transcription. Similarly, deletion of the 15 C-terminal amino acids of Spo0A prevents sporulation and two single point mutations, A257V and A257E, result in proteins that retain their DNA binding ability but do not support sporulation (Ferrari et al., 1985; Perego et al., 1991).

Thus, the possibility of an interaction between Spo0A and the polymerase alpha subunits at the spoIIG promoter was explored.

RNA polymerase reconstituted with either wild type α subunits or α subunits deleted by 15 or 59 amino acids from the C-terminus were equally stimulated by activated Spo0A (Figure 30). Commensurate with this stimulation, the wild type and mutant polymerases appeared to use the same initiation mechanism (Figure 31A). However, the maximum level of transcription by holoenzyme reconstituted with the α mutants was decreased and some differences in the structure of open complexes formed by RNA polymerase containing the alpha mutants were noted (Figures 31A, B and 32).

The three dimensional structure of E. coli RNA polymerase shows a potential DNA-binding channel that could accommodate approximately 16 bp of DNA (Darst et al., 1989). Because RNA polymerase generally protects 60 bp or more at promoters, it has been suggested that one step in promoter melting may involve wrapping of the promoter DNA around RNA polymerase. The impetus for this wrapping may be due in part to contacts between the α subunit of RNA polymerase and the DNA. As demonstrated for the E. coli alpha subunit (Ross
Figure 35  Amino acid sequence of the C-terminal domain of Spo0A. The amino acid sequence of Spo0A (Ferrari et al., 1985) from positions 110 to 267 is shown. The diamond marks the trypsin cleavage site within the linker. Also shown is the amino acid sequence from φ29 p4 protein (Mizukami et al., 1986) and mutations that affect its transcription activation properties (Mencia et al., 1993 and references therein). The positions of the $suv3$ and $suv4$ suppressor mutatations of the spo0A9V mutation and the spo0A153 mutations in Spo0A are indicated (Perego et al., 1991). The spo0AΔ15 mutant described by Ferrari et al. (1985) is also marked. Adapted from Spiegelman et al. (1995).
223 AWSRGNI\textsc{DSISS} LF\textsc{GTYV} SMTKA\textsc{KPTN} NSEF\textsc{IAMV} ADKLRL\textsc{EHKAS}

\textit{V-spo0A9v; E-spo0A153}

\textit{spo0A \textsc{A15}}

\textit{MTKPNTLDWSRIQKPRLERRKSMV}

\textit{gp4}

\textit{Q blocks RNAP binding}
\textit{A reduces RNAP binding}
\textit{deletion to here eliminates activity}
et al., 1993), the alpha subunit of \( B. subtilis \) probably interacts with AT rich UP elements of promoters (Frederick et al., 1995). There is a 5 bp AT rich region directly 5' to the site 2 0A boxes (positions -57 to -61 of the \( spoII\)G promoter). Interaction of the C-terminus of alpha with this region might stabilize a bend in the DNA template and wrap the DNA around the ternary complex to promote transcription initiation. Since DNA molecules with static bends migrate slower than linear molecules, this could explain the lower mobility of the wild type polymerase complex as compared to complexes formed with polymerase containing the mutant alpha subunits (Figure 32). This difference supports the notion that alpha affects the interaction of the polymerase with the \( spoII\)G promoter. The wild type alpha interaction could stabilize the initiated complex, raising the maximum level of transcription and thus supporting an intrinsic role for alpha in transcription initiation. While we do not have direct evidence, it is also possible that the initial complexes formed by the enzymes with the deleted alphas differ because the mutant alphas do not interact with the AT sequence and as a consequence RNA polymerase was misaligned at the promoter.

5.0. Activating regions in \( \sigma^A \), \( \sigma^Le, \) and the C-terminus of Spo0A.

A number of transcription activators from different bacterial species have been demonstrated to increase promoter activity through contacts with the sigma subunit of RNA polymerase (see Introduction). Spo0A is a class II activator, binding to the promoter in a region overlapping the -35 recognition sequence for RNA polymerase (Ishihama, 1993) and is proposed to make contact with the \( \sigma \) subunit (Baldus et al., 1995; Schyns et al., 1997; Buckner et al., 1998; Buckner and Moran, 1998; Hatt and Youngman, 1998). Spo0A is unique among transcriptional regulators in that it is apparently the only positive regulator demonstrated to activate transcription from promoters transcribed by
holoenzyme containing different sigma factors, $\sigma^A$ and $\sigma^H$ (Baldus et al., 1995). Supporting evidence for this conclusion includes the isolation of mutations in both $\sigma^A$ and $\sigma^H$ which prevent transcription from Spo0A-dependent promoters but have no affect on Spo0A-independent promoters (Baldus et al., 1995; Schyns et al., 1997; Buckner and Moran, 1998). These mutations lie within analogous regions of $\sigma^A$ and $\sigma^H$ (region 4) and are likely to be spatially close to Spo0A-P when it is bound at the 0A boxes. Furthermore, mutations in Spo0A that specifically prevent stimulation of the $\sigma^A$-dependent spollG promoter but do not affect transcription from the $\sigma^H$-dependent spollA promoter suggest that Spo0A may have separate contact regions for these sigma factors (Figure 9; Buckner et al., 1998; Hatt and Youngman, 1998). Finally, a mutation in Spo0A has been isolated that restored transcription of the spollG promoter by holoenzyme carrying a mutation in $\sigma^A$ (Buckner et al., 1998). No mutations have been isolated in Spo0A that specifically affect transcription from $\sigma^H$-dependent promoters but have no affect on transcription from $\sigma^A$-dependent promoters.

5.1. A region in Spo0A required for PspollG stimulation.

The classical alanine scanning mutagenesis technique (Wells, 1991) was adapted to probe the extreme C-terminal residues of Spo0A. Valine substitutions were made because i) the original analysis was to include the C-terminal 15 amino acids of which 3 are alanine residues in the wild type protein compared to a single valine; ii) intragenic suppressors of the alanine to valine substitution at position 257 had been isolated (Perego et al., 1991) and this would be useful for future mutagenic studies regarding the internal structure of Spo0A; iii) next to alanine, valine is the most suitable amino acid for negating electrostatic effects while minimizing additional steric effects.
Mutations identified a second region of Spo0A required for activation of the $\sigma^A$-dependent spoIIG promoter. This region spans 4 amino acids in the C-terminus of Spo0A (positions 257, 258 and 260). Valine substitutions at these positions do not adversely affect the conformation or phosphorylation of Spo0A since mutant strains displayed normal $abrB$ regulation. Presumably, the mutants retained the ability to bind DNA and repress $abrB$ expression. The impairment of spoIIG activation was complete and the strains were not differentially defective in their function. The effects of the various mutants could be explained as follows. Alanine is not an amino acid typically involved in direct protein-protein contacts. Presumably, the introduction of a bulky (V; A9V mutant) or charged (E; 153 mutant) substituent at position 257 prevents critical contacts with polymerase by either a steric effect, an electrostatic effect or both. The substitution at L260 can be considered to mimic the classical alanine interference characteristics, namely, to eliminate a potentially important protein-protein interaction due to its shorter side chain. The D258V mutation is difficult to interpret since both a charge and steric effect are introduced by the valine substitution at this position. Overall, these three amino acids resemble the I229, D230 and I232 sequence identified by Hatt and Youngman (1998) as participating in the Spo0A-dependent activation of $\sigma^A$-dependent promoters. This region lies 20 residues away in the primary structure of Spo0A. The crystal structure of Spo0A has not been solved but these two regions may be closely associated in the native protein.
5.2. The possible effects of the region 1 and 2 mutations in SpoOA on $\sigma^A$-dependent transcription.

A stretch of 14 amino acids from residues 227-240 of SpoOA are required for transcriptional activation of $\sigma^A$-dependent promoters but not $\sigma^H$-dependent promoters (Buckner et al., 1998; Hatt and Youngman, 1998). Furthermore, the mutation S231F in SpoOA may establish a new interaction with RNA $\sigma^A$-polymerase since it partially repressed the $\sigma^A$ H359R, H359A and K356E mutations to restore transcription from $spollG$ (Buckner et al., 1998). Thus, it seems likely that region 1 defines a segment of SpoOA involved in direct protein-protein contact with the $\sigma^A$-holoenzyme.

The results of this thesis has led to the identification of a second region in SpoOA required for activation of transcription from $spollG$, residues 257-260. Included in this group is the A257V mutation previously characterized as incapable of stimulating transcription from the $\sigma^H$-dependent $spollA$ promoter. The isolation of two intragenic suppressor mutations of A257V, H162R (suv4) and L174F (suv3; Figure 9) suggests that A257, and possibly D258 and L260, interact with amino acids in the middle of the C-terminus and are involved in maintenance of the activated structure of SpoOA or in positioning of the $\sigma^A$ activating region. A similar role has been assigned to residues in the extreme C-terminus of OmpR which link with central amino acids of the C-terminus to form a compact hydrophobic structure (Kondo et al., 1997). Isolation of additional suppressor mutants of the valine substitutions created in this study may help to better define the central regions within SpoOA that may interact with A257, D258 and L260 and provide further insight into its activation mechanism.
5.3. The potential effect of the region 1 and 2 mutations in SpoOA on $\sigma^H$-dependent transcription.

A region in SpoOA involved specifically in $\sigma^H$-dependent activation has not been defined. The studies of Perego and coworkers (Perego et al., 1991) provide an indication of where such a region might reside. However, the effect of A257V on spoIIIG expression may be indicative of a general 'contact center' role for this region of SpoOA in which contact sites for $\sigma^A$ and $\sigma^H$ overlap and are not mutually exclusive. The p4 protein provides an example of a single amino acid, A120, that participates in both activation and repression of target promoters (Monsalve et al., 1996b), although, this residue may play a larger, more general structural role in activation (see section 5.2). The effects of the D258V and L260V mutations on expression from the $\sigma^H$-dependent spoIIA promoter are being investigated. In light of the complexity of activation by CAP dimers at Class II promoters (Introduction), it is possible that a separate $\sigma^H$-specific contact site may lie elsewhere in the protein.

6.0. Potential consequences of phosphorylation on Spo0A.

The mechanisms of stimulation of initiation at $\text{P}_{\text{spolIG}}$ by Spo0A-P and Spo0ABD are similar but they are not identical. The data of Figures 21 and 22 indicated that Spo0A-P plays an additional role in stabilizing the uninitiated complex. The differential effect of chloride and acetate anions on transcription suggested that the transcription complex was stabilized through protein-protein contacts between Spo0A-P monomers and/or Spo0A-P and the RNA polymerase.
6.1. Effects of phosphorylation on DNA binding.

Previous reports have suggested that phosphorylation stimulated transcription from the spolIG promoter by enhancing binding to weak OA boxes (Satola et al., 1992; Baldus et al., 1994). Experiments reported here revealed that phosphorylation enhanced binding of SpoA–P to the OA boxes relative to SpoOA (4 fold increase). However, no increase in binding was observed for SpoOABD relative to SpoOA, although it was able to stimulate both the initial rate of formation and maximum levels of heparin-resistant complexes at PspolIG far more efficiently than SpoOA. Thus, stimulation of transcription from PspolIG was not due to enhanced DNA binding per se, but appeared to be a consequence of some other effect of phosphorylation. The presence of OA recognition sequences in the promoter regions of genes activated by this response regulator indicates that DNA binding is an important feature of stimulation. The OA boxes may function to orient the activator for co-operative binding or simply increase the local concentration of SpoOA for interaction with the RNA polymerase. One explanation for the enhanced binding by SpoOA upon phosphorylation may arise from the observation that OA boxes usually occur as pairs and that two SpoOA proteins appear to bind to paired OA boxes simultaneously (Satola et al., 1992; Bird et al., 1993; Baldus et al., 1994). Phosphorylation may expose sites on the N terminus that interact when the two proteins bind to adjacent OA boxes. This could drive SpoA–P to bind to weak OA boxes. OmpR shows similar "two at a time binding" (Harlocker et al., 1995) and phosphorylation increases cooperative binding to OmpR boxes (Huang et al., 1997).

The stronger binding of SpoA–P compared to SpoOABD (Figure 23) mirrored the increase in relative stability of transcription complexes containing SpoA–P compared to those containing SpoOABD (Figure 21). Thus, the
stronger binding by Spo0A–P could account for the increase in stabilization of the transcription complex. However, the ability of Spo0A–P to stimulate the initial rate of transcription (greater than 50 fold; Bird et al., 1993; Bird, 1995; Bird et al., 1996) cannot be accounted for by the stronger binding of Spo0A–P for DNA.

6.2. The hydrophobic effect.

The observation that phosphorylation of the N-terminus of Spo0A contributed to the stability of the ternary complex to challenge by organic ions (the hydrophobic effect) can be explained as follows. The data of Arakawa and Timasheff (1984a, b) indicate that, relative to water, acetate and glutamate anions are preferentially excluded from hydrophobic regions on the surface of proteins, creating an anion gradient. Increasing the overall salt concentration potentiated this effect and results in the entropically unfavorable formation of a water lattice at the protein surface. This unfavorable condition can be eliminated by protein association to shield these hydrophobic residues. The shielding of hydrophobic residues blocks anion influences on the protein which, if the anion inhibited activity, would be observed as resistance to organic but not inorganic anions (Leirmo and Record, 1990).

The hydrophobic effect implies that it is possible to bury the hydrophobic residues. The N-terminal domain of Spo0A is highly similar to that of the CheY protein of E. coli (Figure 36). CheY is one of the best understood of the receiver domain family of two component signal transduction systems. CheY is a single domain response regulator that interacts with at least three different proteins, CheA, CheZ and FliM. CheY is phosphorylated by the sensor kinase CheA in response to changes in extracellular chemical concentrations. Phosphorylation activates CheY by inducing a conformational change that enhances its binding
to the FlIM protein at the flagellar motor. This results in a switch in flagellar rotation from counterclockwise to clockwise. CheY is deactivated by an intrinsic autophosphatase activity and CheZ (for review see Stock and Surette, 1995).

The NMR and crystal structure of CheY have been reported (Stock, A. M. et al., 1989; Voltz and Matsumura, 1991; Stock et al., 1993; Bellsolell et al., 1996). A large number of residues near the site of phosphorylation in CheY (D57) exhibit a significant structural shift change upon phosphorylation of CheY. The amino acids that become shifted when CheY is activated include D57, M60, M63, D64, G65, L66, E67, L68 and L69 (Figure 36). There is an almost identical sequence near the site of phosphorylation in SpoOA, namely D56, L62, D63, G64, L65, A66, V67 and L68. Large conformational changes at the site of phosphorylation in CheY extend to distant residues (Drake et al., 1993). One such residue is the tyrosine at position 106 of CheY which has been proposed to play an important role in the signalling process. The Y106 side chain can occupy two orientations, either a solvent exposed outside position or a solvent inaccessible inside position (Voltz and Matsumura, 1991). In the active state, Y106 of CheY~P is packed in the interior of a hydrophobic pocket formed by residues W58, M85, T87, I95 and A98 (Zhu et al., 1997a). In Spo0A, the corresponding amino acids are Y104 (which 'flip' like Y106 of CheY), I57, M84, T86, T94 and A97. In addition, residues F111 and T112 of CheY are part of the β5-α5 loop located on the surface of CheY that participates in its interaction with the CheZ protein (Zhu et al., 1997b). K109 of CheY~P is packed against M17, V86 and A88 and is proposed to slide forward during activation, dragging the β5-α5 loop and triggering larger movements at the functional surface of the protein (Bellsolell et al., 1996). Again, these positions in Spo0A, F110, D111, K108, V16, L85 and A87 are similar.
Figure 36  Comparison of the N-terminal sequences of Spo0A and CheY.

Positions of α-helices (boxed) and β-strands (underlined) for CheY are from crystal structure. Positions in Spo0A (β-strands, dotted line and α-helix, dotted box) are predicted. Amino acid positions for Spo0A are marked by the plain text and those for CheY are in bold. Closed circles mark the position of the hydrophobic pocket on CheY and open circles mark a similar region in Spo0A. The sites of phosphorylation in Spo0A (D56) and CheY (D57) are marked by the (*).
Grimsley et al., (1994) analyzed the protease sensitivity of Spo0A in solution and bound to DNA. It was demonstrated that the cleavage pattern of Spo0A was altered from a central to an N-terminal site when bound to DNA. This was suggestive of a conformational change in each Spo0A upon activation. Phosphorylation has been reported to expose hydrophobic residues on CheY (Lowry et al., 1994) and may do the same on the N-terminus of Spo0A. This region could provide the hydrophobic effect seen with Spo0A–P, while unphosphorylated Spo0A or Spo0ABD (which lacks the N-terminus) would not show a difference in salt sensitivity between inorganic and organic salts. Thus, phosphorylation of Spo0A may produce similar effects as in CheY and provide a contact surface for interaction between the N-termini of Spo0A–P monomers or Spo0A–P and RNA polymerase.

6.3. Dimerization of Spo0A–P.

Phosphorylation of Spo0A could induce Spo0A–P self association leading to dimer formation as suggested by Kobayashi et al. for Spo0A–P stimulation of the spo0F promoter (Asayama et al., 1995), and for activation by other response regulators (Minchin et al., 1988; Aiba et al., 1989; Weiss et al., 1991; Porter et al., 1993; Huang et al., 1997). The capacity of the N-terminus to dimerize could be investigated by fusion of the N-terminal domain of Spo0A to the DNA binding domain of λ repressor and measuring activity from the λP_R or λP_RM promoters since only dimeric protein forms are capable of regulating promoter activity. An analogous system has been used to study dimer formation of NtrC and PhoB (Fiedler and Weiss, 1995).
6.4. A proposal for Spo0A-P interaction with RNA polymerase.

Spo0ABD activates transcription yet binds DNA poorly. Furthermore, the N-terminal domain of Spo0A can apparently form dimers in solution\(^1\). Since Spo0ABD lacks the N-terminus, presumably it cannot form dimers. Hence, these properties of Spo0A-P are likely not the critical changes induced by phosphorylation that are necessary for stimulation of the \textit{spollG} promoter. The results of this study and those of Bird \textit{et al.} (1995) support the hypothesis that activation of Spo0A ultimately results in the exposure of a RNA polymerase interaction domain within the C-terminus of the regulator. This site is probably hidden by the N-terminal domain in the non-phosphorylated protein. The C-terminal domain of OmpR contains an exposed loop which is a candidate for interaction with the polymerase because it is the site of mutations affecting polymerase activation (Kondo \textit{et al.}, 1997; Martinez-Hackert and Stock, 1997b). The structure of Spo0A is not known at the moment, but if there is a similar loop, it could well be buried in the non-phosphorylated protein. In this scenario, exposure of a RNA polymerase interaction domain within Spo0A would be sufficient to activate transcription from \textit{PspollG}.

7.0. Negative regulators of initiation.

The process of sporulation is intimately linked to many cellular cycles, including DNA replication. Delicate timing is necessary to ensure that both the mother and forespore receive a chromosome before septation is complete (Parker \textit{et al.}, 1996). Furthermore, the inappropriate expression of the \textit{spollG} and \textit{spollA} operons is toxic and transcription from these operons is tightly regulated \textit{in vivo} (Errington, 1993). Several negative regulators of sporulation have been identified genetically. The activities of two of these, Spo0JA and

\(^{1}\)R. J. Lewis and J. A. Brannigan, York University, UK (personal communication).
SinR, have been characterized in vitro. They represent two very different mechanisms for negatively regulating expression from the *spollG* promoter.

7.1. SpoOJA.

Chromosome replication has been shown to influence sporulation, but the link between the two processes is unknown. Two of the proteins involved in replication of the chromosome are SpoOJA and SpoOJB (for review see Errington, 1993; Hoch, 1998). The *spoOJA* and *spoOJB* genes reside within the same operon (Mysliwiec *et al.*, 1991; Ireton *et al.*, 1994). *spoOJA* and *SpoOJB* are homologues of the ParA/ParB family of plasmid proteins that are required for the proper partitioning of plasmids to daughter cells during replication (Hiraga, 1992; Hayes *et al.*, 1994). Deletion of the *spoOJB* gene in *B. subtilis* generates a sporulation negative phenotype and these strains did not express the stage II genes (Ireton *et al.*, 1994). Deletion of both *spoOJA* and *spoOJB* reestablishes the sporulation proficient phenotype. This suggested that SpoOJA was an inhibitor of sporulation and stage II gene expression and that SpoOJB repressed the activity of SpoOJA.

Biochemical analysis of the inhibitory mechanism of SpoOJA at the *spollG* promoter has been carried out in our lab (Cervin *et al.*, 1998). Recombinant SpoOJA could not bind double stranded DNA on its own or with SpoOA−P. It also had no effect on the binding of RNA polymerase to the promoter. However, when SpoOJA was added to initiated SpoOA−P-RNA polymerase-promoter complexes, the complex was disrupted. In looking for a possible mechanism to explain this effect, it was found that SpoOJA binds to single-strand DNA. Thus, creation of the initiation bubble by the coordinated efforts of SpoOA−P and RNA polymerase may provide the DNA target for
Spo0JA. The bubble templates created in this study could be used to better define the recognition sequence for Spo0JA.

7.2. SinR.

SinR was originally identified by its sporulation deficient phenotype when present in multiple copies (Mandic-Mulec et al., 1992; Bai et al., 1993). In vitro studies in our lab have demonstrated that SinR binds double stranded DNA and recognizes specific sequences in the spolIG promoter. It inhibits transcription by preventing open complex formation without displacing polymerase from the promoter. This repressor can prevent spolIG transcription at any point prior to formation of an initiated complex. Activation requires the displacement of SinR by Spo0A–P (Cervin et al., 1998).

8.0 Unanswered questions.

Despite the expanse of information detailing the pathway of signal transduction and the activation of transcription for the two-component system governing the sporulation process in B. subtilis, many key aspects of the reaction mechanism remain poorly understood. Some of these are discussed below.

8.1 Reconciling the effects of phosphorylation on stimulation of σA and σH-dependent transcription.

The OA boxes in σA-regulated promoters are generally in a different orientation and location to those in σH-regulated promoters (Spiegelman et al., 1995). Thus, it is difficult to envision how Spo0A–P could contact the homologous region of both sigma factors at respective promoters since Spo0A–P would be positioned differently. The length of DNA protected from
DNAse I digestion by Spo0A−P is not uniform at all 0A boxes. At some promoters, a single 0A box can lead to protection of as little as 16 bp or as much as 40 bp (Spiegelman et al., 1995). This suggests that the protein can oligomerize at some sites even when only a single binding site is present. At promoters in which the 0A boxes appear in one orientation, for example at σH-dependent promoters, oligomerization could induce DNA bending at to allow Spo0A−P to contact RNA polymerase at the start site. At other promoters where the 0A boxes are in the opposite orientation, for example at σA-dependent promoters, Spo0A−P may already be correctly positioned to contact RNA polymerase and significant DNA bending might not be necessary. In this way, a single contact site on Spo0A could stimulate transcription from two types of promoters. Alternatively, the σA and σH contact sites in Spo0A−P may be in different regions and the direction and location of the 0A boxes serves to correctly position Spo0A−P to contact RNA polymerase. One might test these possibilities by flipping the 0A boxes in the spolIG promoter and assessing response to stimulation by Spo0A−P. Detailed analysis of σH-dependent transcription by Spo0A−P has not been carried out and this information would be useful.

8.2. N-terminal inhibition of the output domain.

Genetic and biochemical experiments have led to the conclusion that the transcription activation functions of Spo0A reside within the C-terminal domain and that these functions are inhibited by the N-terminal domain. In this respect, Spo0A is like many other members of the response regulator family. However, it is clear from these studies that the N-terminus does not sterically hinder the interaction of the HTH DNA binding motif with DNA and activation of Spo0A does not simply involve changing the relationship of the domains to expose the
DNA binding site as proposed for NarL (Baikalov et al., 1996). The precise nature of the switch from an inactive to an active state has not yet been elucidated. Two plausible events could give rise to the activated state. Phosphorylation could change the spatial location of amino acids in the receiver domain which are required for inhibition of the output domain or the receiver and output domains could compete for a site on the polymerase and phosphorylation on the N-terminus might inhibit its binding to RNA polymerase. Understanding transcription activation by Spo0A would be significantly advanced if the structure of the C-terminal domain and the intact protein can be determined.

8.3. Identification of the DNA binding region of Spo0A.

The position of the DNA recognition motif of Spo0A was proposed based on characterization of spo0A homologues from several Bacillus and Clostridium species (Brown et al., 1994). However, it has never been demonstrated to be the actual DNA-binding site. Mutational analysis needs to be conducted to define the amino acids involved in DNA recognition.

8.4. Regulation of spoIIG transcription by the promoter structure.

The barrier to transcription from the spoIIG promoter is clearly related to the inability of RNA polymerase to denature the DNA in the -10 region. This stems from the unusual positioning of the sigma recognition sequences. However, a detectable amount of specific transcription from the spoIIG promoter is routinely observed in vitro. How is this possible? First, this observation suggests that negative regulators participate in the control of transcription from spoIIG (see section 7). Second, it indicates that a small probability exists for holoenzyme to establish effective contact with the -10 region. Although the
initial binding of holoenzyme to the promoter is mediated by contacts with the -35 region, it has been demonstrated that the upstream half of the -10 region is important for this binding (Dombroski et al., 1992; Dombroski et al., 1996; Dombroski, 1997). This potential may be increased by mutation of the spolIG promoter to include an 'extended -10' sequence which may obviate the need for Spo0A\textsuperscript{A--P}. Likewise, decreasing the spacer length to 17 bp might also bypass the activator requirement. These experiments are currently underway\textsuperscript{2}. The contribution of individual OA boxes to stimulation is also of interest. Activation of Spo0A-dependent promoters by a single OA box in the vicinity of the -35 region of the promoter has been proposed at spolIE (York et al., 1992) and recent studies in our lab suggest that of the four OA boxes within the spolIG promoter, only the most downstream OA box is absolutely required for stimulation\textsuperscript{3}. This suggests that only a single OA box is sufficient for activation of transcription. If all four OA boxes were destroyed, it might be that Spo0A could activate transcription from solution at elevated concentrations. This ability has been observed for NtrC (North and Kustu, 1997), NifA (Buck and Cannon, 1989), DctD (Huala et al., 1992) and N4SSB (Miller et al., 1997).

8.5. DNA untwisting.

The results of Bird et al. (1996) suggest that Spo0A may make transient contacts with RNA polymerase to allow distortion of the DNA, possibly by untwisting it. The untwist-and-melt model proposed by Stefano and Gralla (Stefano and Gralla, 1982) postulates that the spacer DNA plays an active role in open complex formation by providing a conduit for the conversion of RNA polymerase binding energy into DNA distortion energy which helps to initiate...

\textsuperscript{2}V. Mendoza, D. A. Rowe-Magnus and G. B. Spiegelman, University of British Columbia, Can.
\textsuperscript{3}V. Mendoza, D. A. Rowe-Magnus and G. B. Spiegelman, University of British Columbia, Can. (unpublished data).
the DNA melting process. Ayers et al. (1989) used heteroduplex templates to investigate the contribution of DNA untwisting to open complex formation. The heteroduplex was made by isolating and annealing 3 single-strand DNAs so that one strand was continuous while the other (made from two DNA molecules) contained a specific nick. These templates, in which one DNA strand is nicked, would eliminate any contribution of torsional energy to DNA melting. In the case of E. coli RNA polymerase, such a template is an effective target for binding but not for initiation (Ayers et al., 1989) Such experiments are currently underway4 for the spollG promoter.

8.6. Open complex formation at spollG as a tool to study the beginnings of DNA strand separation.

The mechanism for DNA strand melting by RNA polymerase is unknown. The stability of the partially denatured DNA strands at the spollG promoter is a fortuitous characteristic. The separation of the two steps may provide a useful experimental tool to define the contributions of SpoOA−P and the sigma subunit to this strand separation process. In vitro analysis of the mutations in Spo0A isolated in this study may be very informative in this regard. Mutations within Spo0A or in the position of the 0A boxes that alter transcription may cripple the denaturation step and could be used to dissect the mechanism behind DNA melting which, as has been pointed out (Busby and Ebright, 1997; Li et al., 1997), is one of the mysteries of transcription initiation.

4V. Mendoza, D. A. Rowe-Magnus and G. B. Spiegelman, University of British Columbia, Can.
REFERENCES.


Reitzer, L. J. and Magasanik, B. (1986). Transcription of \textit{glnA} in \textit{E. coli} is stimulated by activator bound to sites far from the promoter. Cell 45, 785-792.


