INVESTIGATION OF THE TRANSCRIPTION ACTIVATING PROPERTIES OF SPO0A FROM BACILLUS SUBTILIS

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Abstract

Spo0A is a key regulator of sporulation in *Bacillus subtilis*. Genetic investigations have indicated that Spo0A activates or represses transcription of various loci which ultimately commit the cell to spore development under poor nutrient conditions. The transcription regulating properties of Spo0A are believed to be controlled by protein phosphorylation through a signal transduction system termed a 'phosphorelay'. The *spoIIA* and *spoIIG* operons are two targets for activation by Spo0A-P that are essential to the induction of a sporulation response because each encodes a sporulation specific sigma factor.

An *in vitro* transcription assay was used to examine the effect of Spo0A and its phosphorylation on transcription initiation at the *spoIIA* and *spoIIG* promoters (*P*_{spoIIA} and *P*_{spoIIG}). Phosphorylation of Spo0A dramatically enhanced its ability to stimulate the level of transcription initiation at both promoters. A kinetic analysis of the initiation process at *P*_{spoIIG} demonstrated that the rate of initiation was independent of the concentration of RNA polymerase and promoter template. In contrast, increased concentrations of Spo0A-P accelerated the initiation reaction at *P*_{spoIIG} by affecting a rate limiting step that occurred after RNA polymerase bound to the promoter. Initiation rates at *P*_{spoIIG} were also stimulated by a truncated form of the Spo0A protein (Spo0A^BD) which contained the DNA binding domain but not the phospho-acceptor site. While transcription stimulation by Spo0A or Spo0A^BD was observed to be extremely salt sensitive, the effect of Spo0A-P was insensitive to salt concentration if the predominant anion was acetate.

DNaseI protection experiments demonstrated that both Spo0A and Spo0A-P catalyzed structural changes in ternary complexes formed between RNA polymerase and *P*_{spoIIG}. These isomerizations appeared to correlate with the modest and dramatic effects of Spo0A and Spo0A-P, respectively, on *P*_{spoIIG} transcription. DNaseI
protection patterns obtained at various temperatures showed that although protein binding appeared normal, low temperature prohibited the structural transformations attributed to Spo0A(-P). Footprinting experiments carried out on a mutant promoter construct (P_{spoII}^{M94/84}) indicated that Spo0A binding sites located at -94 and -84 relative to the transcription start site, are dispensable to the effect of Spo0A(-P) on ternary complex isomerization. However, these sites may function to enhance factor binding to what seem to be the crucial 0A boxes at -50 and -40.

The biochemical analysis presented in this thesis supports the hypothesis that the phosphorelay functions as a signal transduction system that can induce a sporulation response through activation of the transcription regulating properties of Spo0A.
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**Abbreviations and symbols**

Asp  aspartate.
bp    Base pair.
C_I   RNA polymerase/P_{spolIIG} ternary complex.
C_{II} RNA polymerase/Spo0A/P_{spolIIG} ternary complex.
C_{III} RNA polymerase/Spo0A-P/P_{spolIIG} ternary complex.
Cl^-  chloride ion.
HEPES 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid.
HR  heparin-resistant RNA polymerase/promoter complex.
HS  heparin-sensitive RNA polymerase/promoter complex.
k_f  forward isomerization rate constant.
k_r  reverse isomerization constant.
k_1  forward rate constant for the formation of a polymerase/promoter complex.
k_{-1}  rate constant for the dissociation of a polymerase/promoter complex.
k_{obs} observed overall rate of heparin-resistant complex formation.
KAc  potassium acetate.
KCl  potassium chloride.
kDa  kilodalton.
NaCl sodium chloride.
NTPs nucleotide triphosphates.
PAGE polyacrylamide gel electrophoresis.
PA2 A2 promoter from the Bacillus phage, φ29.
P_{spolIA} promoter from the spolIIA operon of Bacillus subtilis.
P_{spolIIG} promoter from the spolIIG operon of Bacillus subtilis.
SDS sodium dodecyl sulfate.
Spo0A-P phosphorylated form of Spo0A protein.
(1/τ) overall (pseudo first-order) rate constant for heparin-resistant complex formation.
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Dedication

This thesis is dedicated to my children, Nathan and Katrina.
**Introduction**

I. Sporulation in *Bacillus subtilis*.

1. Sporulation as a response to starvation.

   In the natural environment, microbial growth is often prohibited by the depletion of limited nutrient resources. For the Gram-positive soil bacterium, *Bacillus subtilis*, nutrient exhaustion may lead to prolonged periods of starvation. In this event, cells cease to grow vegetatively and differentiate into metabolically inert endospores. Mature spores are characterized by resistance to extremes in temperature, pH and dehydration, and will germinate once they encounter favorable growing conditions. Because each differentiating cell yields a single spore, the sporulation process is not considered to be a form of reproduction but rather a strategy for coping with a barren environment.

   Sporulation is generally accepted to be one of the most comprehensive examples of cell differentiation among prokaryotes. The process features a continuum of transformations in cell physiology, morphology and biochemistry, and is accompanied by remarkable temporal and spatial coordination of gene expression (for reviews see Piggot *et al.*, 1981; Losick *et al.*, 1984; Errington, 1993). For this reason, sporulation has attracted the interest of microbiologists and developmental biologists for many years (Keynan *et al.*, 1984). *Bacillus subtilis* has been the most intensively studied of the endospore forming bacteria as it is particularly amenable to genetic analysis (Youngman *et al.*, 1989). Genetic and molecular biology techniques have been exploited to isolate genes essential to sporulation (*spo* genes) and identify the functions of the proteins they encode. Accordingly, the organism has provided important insights especially into regulatory mechanisms that govern the expression of developmental genes in differentiating bacteria.
2. The morphology of sporulation in *B. subtilis*.

Sporulation in *B. subtilis* is characterized by a series of distinctive morphological structures which are depicted in Figure 1. The appearance of specific structures has been used to divide the progression of spore formation into stages of development (Ryter, 1965; Losick *et al.*, 1986; Errington, 1993). While cells growing vegetatively are defined as stage 0, it was originally thought that stage I began with the cessation of exponential growth. However, no mutations have been isolated which block sporulation at this point in development. Therefore, stage I is no longer considered to be unique to sporulation (Piggot and Coote, 1976). It is now postulated that *B. subtilis* enters into a stationary phase or 'transition-state' where cells may acquire new traits as they attempt to adjust to poor nutrient conditions without initiating a sporulation response. These include motility, competency and the ability to secrete proteases, nucleases and antibiotics. Given the time and resources devoted to spore formation it is presumed that inappropriate induction of sporulation would have serious repercussions for *B. subtilis* by placing the organism at a competitive disadvantage. Only in the event of severe or prolonged starvation will a sporulation response be initiated.

The first conspicuous structure uniquely associated with sporogenesis is the formation of an asymmetrically positioned septum that divides the cell laterally into two compartments of unequal sizes (stage II) (Hitchins and Slepecky, 1969). Completion of the septum separates the forespore (small compartment) from the mother cell (large compartment). Each compartment contains an intact copy of the chromosome and from this point patterns of gene expression in the forespore and mother cell begin to diverge (Errington *et al.*, 1990; Margolis *et al.*, 1991). Spore development continues with a migration of the membrane surrounding the cytoplasm of the mother cell, toward the pole of the forespore. Eventually, the forespore is completely engulfed within a double membrane of opposite polarities (stage III). The space between the two membranes becomes the site of cortex synthesis (stage IV). The cortex consists of a cell wall
This cartoon depicts the various morphologies that characterize endospore development in *B. subtilis* (Introduction II, 2). Beginning with the vegetative state (defined as stage 0), the sporulation response can only be initiated after DNA replication has been completed. The first distinct structure becomes evident with formation of an asymmetric septum (stage II). Following complete engulfment of the forespore (stage III), the spore cortex (stage IV) and coat (stage V) are synthesized. Once the spore has undergone maturation (stage VI) it is released through lysis of the mother cell (stage VII).

*a* Adapted from Losick *et al.*, 1986.
material resembling a loosely cross linked form of peptidoglycan (Warth and Strominger, 1972) and is believed to contribute to the heat resistance of mature endospores (Gould, 1984).

A tough spore exterior is created with coat proteins that are synthesized within the mother cell and deposited on the outside surface of the forespore (stage V) (Jenkinson et al., 1981). At least twelve different spore coat proteins have been identified and since the formation of the spore coat occurs in the absence of protein synthesis it has been proposed that the coat proteins are arranged by self-assembly processes (Jenkinson et al., 1980). Stage VI is associated with maturation of the forespore. It is marked by dormancy and the acquisition of most of the distinguishing properties of an endospore, including resistance to UV radiation, dessication, heat and organic solvents (Dion and Mandelstam, 1980; Jenkinson et al., 1980; Gould, 1984). Sporulation culminates with the release of a mature spore through mother cell lysis (stage VII) approximately 8 - 10 hours after the process begins.

3. Sporulation genes.

Classical genetic techniques have identified over 50 spo genes which are scattered in four loosely defined clusters around the B. subtilis chromosome (Piggot et al., 1990). Mutations within a spo gene arrest spore formation prior to the stage in development that depends on the function of its gene product. Consequently, spo gene mutations are easily classified because their phenotypes are often manifested in developmental pathways that are blocked at a particular morphology. For instance, stage II mutations may permit normal asymmetric septation (stage II) but prevent the subsequent engulfment of the forespore (stage III).

Studies which test the expression of spo-lacZ fusions in different spo backgrounds have elucidated dependence patterns and expression times for many spo genes (Zuber and Losick, 1983). Currently, 12 dependence classes of spo genes have been distinguished based on similarities in time and location of their expression and
comparable patterns of epistasis (Errington, 1993). A major accomplishment of this study has been the identification of various regulatory genes that were discriminated by their involvement in the expression of spo genes within a single dependence class.

II. Regulation of gene expression during sporulation.

1. Negative and positive regulation of spo genes.

Although there are several examples of post-transcriptional regulation involving elaborate mechanisms of proteolytic processing, most of the regulation of spo gene expression occurs at the level of transcription (Stragier et al., 1988; Healy et al., 1991).

1.a. Negative regulation of sporulation: transition-state regulators.

During exponential growth, the inappropriate expression of many spo genes is prevented by the transcriptional repressors AbrB, Sin, and Hpr. These proteins are also essential for the induction of alternative processes associated with post-exponential growth (Gaur et al. 1986; Perego and Hoch, 1988; Strauch et al., 1989; Kallio et al., 1991). It has been postulated that upon entering the transition-state, AbrB, Sin, and Hpr, function as molecular switches within the cell circuitry to effect the 'decision' to sporulate or adopt an alternate strategy in response to nutritional stress. Accordingly, these proteins have been termed 'transition-state regulators' (Strauch and Hoch, 1993).

1.b. Positive regulation of sporulation: the sigma factor cascade.

Core RNA polymerase from B. subtilis is composed of β, β', α2, ω and δ subunits and retains the ability to catalyze synthesis of RNA transcripts. However, promoter specific initiation of transcription is conferred by the σ subunit which combines with the core polymerase to form the holoenzyme (Helman and Chamberlin, 1988). Thus far, 10 different sigma factors have been isolated from B. subtilis (Moran, 1989; Debarbuille et al., 1991). The use of alternate sigma factors provides efficient regulation of gene expression because each σ subunit directs the polymerase to transcribe genes that have common DNA sequences within their promoters. Usually, these sequences are found at
the -10 and -35 positions with respect to the transcription start-site (Helman and Chamberlin, 1989; Moran, 1988).

During spore formation much of the temporal and spatial regulation of spo gene expression is controlled through an ordered series of σ subunit replacements (Losick and Pero, 1981; Stragier and Losick, 1990). Each replacement changes the promoter specificity of RNA polymerase and effectively reorganizes global regulation of gene expression. At least six different σ subunits are involved in the 'sigma factor cascade', each directing the transcription of a subset of spo genes at particular times and locations. The first spo genes (the spo0 and spoII genes), are transcribed by RNA polymerase containing σA (EσA), the predominant sigma factor in vegetative cells (Kenney and Moran, 1991; Kenney et al., 1991), or σH (EσH), which is encoded by the spo0H gene and is maximally expressed in stationary phase (Carter and Moran, 1986; Dubnau et al., 1987; Dubnau et al., 1988; Predich et al., 1992). Although originally defined as sporulation specific, EσH is now recognized to be involved in the transcription of genes not directly involved in sporogenesis (Albano et al., 1987; Sonenshein, 1989).

The first σ replacements occur with the appearance of σE and σF, encoded by the spoIIA and spoIIG operons, respectively (Moran, 1989; Stragier and Losick, 1990). They represent the first sporulation specific sigma factors and although both operons are expressed prior to the completion of septation (Gholamhoseinian and Piggot, 1989), σE activity is localized to the mother cell while σF directs transcription from within the forespore (Partridge et al., 1991; Errington and Il ling, 1992). It has been proposed that mechanisms that control σE and σF activities are somehow linked to engulfment of the forespore (Stragier et al., 1988; Stragier, 1989; Losick and Stragier, 1992). Apparently each σ subunit dictates the expression of spo genes in the early development of their respective compartments.

The last of the developmental sigma factors are, σG and σK. Like their predecessors, they direct cell-specific transcription. The σG protein is active in the forespore (Sun et
and $\sigma^K$ governs synthesis of the coat proteins from within the cytoplasm of the mother cell (Kroos et al., 1989). Therefore, these $\sigma$ subunits are responsible for the expression of spo genes involved in the final stages of forespore construction and maturation (Zheng and Losick, 1990).

III. Initiation of the sporulation response.

1. Conditions required for sporulation.

* Bacillus subtilis* cells sporulate when starved for carbon, phosphate or nitrogen but the response is subject to catabolite repression such that an adequate carbon source will prevent sporulation should either of the other two nutrients become limiting (Schaeffer et al., 1965; Freese, 1981; Sonenshein, 1989). It is presumed that starvation leads to the accumulation of a metabolite within the cell that signals the necessity of a sporulation response, however, the nature of this molecule has not been determined. It is known that the intracellular concentration of GTP falls dramatically at about the time sporulation is initiated (Lopez et al., 1981; Freese, 1981) and that decoyinine, an antibiotic that interferes with GTP synthesis, can be used to induce a *B. subtilis* culture to sporulate even in rich media (Mitani et al., 1977).

Under natural conditions, efficient sporulation within a *B. subtilis* population requires high cell density (Grossman and Losick, 1988). There is now good evidence that an extracellular factor, apparently an oligopeptide, is secreted and probably functions as a pheromone-like substance to communicate a sporulation signal between cells (Perego et al., 1991a; Rudner et al., 1991). A second precondition for the initiation of sporulation is that it must be coordinated with the cell cycle to ensure that each sporulating cell contains two copies of the chromosome (Hitchins and Slepecky, 1969; Mandelstam and Higgs, 1974; Dunn et al., 1978), one to be packaged within the spore, and the other to provide a template for gene expression in the mother cell.
2. Genes that regulate the initiation of a sporulation response.

The successive expression of alternative sigma factors provides effective temporal regulation of gene activation during development (Introduction II, 1.b). However, the initiation of a sporulation response cannot be accommodated by a sigma factor cascade because the first inducible spo genes must be transcribed by polymerase already transcriptionally active in vegetative cells. Therefore, some other mechanism must activate the early spo genes, including those encoding the first developmental σ subunits that will commit starving cells to the sporulation pathway.

Currently, there are nine loci which have been designated as spoO (spoOA, spoOB, spoOE, spoOF, spoOH, spoOI, spoOK, spoOL and spoOP) on the basis that mutations within these genes block the induction of sporulation (Hoch, 1976; Errington, 1993). All spo0 genes are maximally expressed during exponential growth, or become fully activated shortly after cells enter stationary phase. Of these genes, spoOA was identified as a key regulator of the transition-state because of the highly pleiotropic nature of the spoOA phenotype (Hoch, 1976). No suppressors of spoOA deletion mutations have been isolated. However, several mutations in spoOA were found to suppress the sporulation defective phenotypes of other spo0 genes including spoOB and spoOF (Hoch et al., 1985; Spiegelman et al., 1990). These observations suggested that Spo0A was indispensable to the induction of sporulation and that its regulating activities were probably modulated by the products of at least some of the other spo0 genes. Elucidation of the exact roles of each of the spo0 genes became possible only after they were cloned and sequenced.

3. Sensing the sporulation signal(s).

Sequencing of the spoOA gene revealed that its product was homologous to a group of proteins collectively known as response regulators (Ferrari et al., 1985; Kudoh et al., 1985). Most of these proteins are transcription regulators which control activation of genes within a tightly coordinated regulon. All response regulators are paired with a second type of protein called sensor/kinases to form two component regulatory systems
which are pervasive in bacteria (for reviews see Kofoid and Parkinson, 1988; Stock et al., 1989; Stock et al., 1990; Bourret et al., 1991). These systems direct appropriate cellular responses to specific environmental changes. Adaptations may be as diverse as the control of chemotaxis, induction of virulence factors, adjustment to fluctuations in osmolarity, switches in the utilization of alternative nutrient sources, or even modifications to cell morphology (Stock et al., 1989). Yet despite this diversity, two component systems share a commonality in the way they sense and direct adaptive response through the use of protein phosphorylation as a mechanism of signal transduction.

All sensor/kinases of two component systems have the ability to perceive environmental change although each is tuned to a separate aspect of the cell's surroundings. While some are cytoplasmic proteins, many span the cytoplasmic membrane to monitor the external environment. Should it detect a significant change, a sensor/kinase will catalyse the transfer of a phosphoryl group from ATP to a highly conserved histidine residue located in its C-terminus (Hess et al., 1988). The sensor/kinase will then transfer the same phosphate moiety to its cognate response regulator. This activates the regulatory properties of the response regulator and allows it to direct an appropriate cellular response.

As a class, response regulators are characterized by significant amino acid identity (typically 20% or greater) throughout their N-terminus domains (Stock et al., 1989). This portion of the protein interacts with the sensor/kinase and contains the phospho-acceptor site which is invariably an aspartate residue. Similarity between C-terminal regions of response regulators is variable but has permitted many to be grouped into subfamilies (Ronson et al., 1987; Stock et al., 1989). Response regulators which mediate an adaptive response by effecting gene expression contain DNA binding domains within their C-terminus which allow them to interact with their designated targets. However, the DNA binding and transcription modulating properties of these
proteins are activated only after their N-terminal domains have been phosphorylated by their sensor/kinase partners (Stock et al., 1989).

4. The phosphorelay signal transduction system.

Once it had been established that Spo0A was a response regulator, a search for its cognate sensor/kinase was undertaken to identify the two component system believed to control the induction of sporulation. This genetic investigation led to the cloning and subsequent biochemical analysis of several recombinant spo0 proteins resulting in the discovery of the 'phosphorelay'. The phosphorelay comprises a signal transduction system that is an unprecedented departure from conventional two component regulatory mechanisms (Burbulys et al., 1991).

The transcription regulating activities of Spo0A appear to be controlled by at least two (KinA and KinB) and possibly more sensor/kinases (Perego et al., 1989; Antoniewski et al., 1990; Trach and Hoch, 1993; Hoch, 1993). Individually, mutations within kinA or kinB delay a sporulation response or decrease the level of spore formation in a population of B. subtilis cells. Only double mutations reduce sporulation to zero leaving cells locked in the vegetative state. While KinA is a soluble protein, it has been determined that KinB spans the cytoplasmic membrane with its sensor domain positioned outside the cell (Trach and Hoch, 1993). Thus, it appears that both intracellular and external factors are crucial to the induction of a sporulation response (Hoch, 1993).

The most remarkable feature of the phosphorelay which thus far is unparalleled among prokaryotes, is that Spo0A is not directly phosphorylated by sensor/kinase proteins. Instead, phosphoryl groups are passed from either KinA-P or KinB-P to Spo0A through two intermediate phosphotransferases, SpoOF and SpoOB (Burbulys et al., 1991). Therefore, the phosphorelay comprises an extended signal transduction system represented by the following schematic:
The entire phosphorelay reaction has been extensively characterized in vitro using recombinant KinA, SpoOF, SpoOB and SpoOA proteins. SpoOF appears to be a truncated version of a response regulator. It is homologous to the N-terminal half of SpoOA (Trach et al., 1985) and contains an aspartate residue which acts as a phosphoacceptor site (Burbuly et al., 1991). SpoOF is a substrate for the kinase activity of KinA and KinB and effectively transfers phosphoryl groups from these proteins to SpoOB. SpoOB displays little homology to two component type proteins but is functionally similar to sensor/kinase proteins in that it transmits a signal to a response regulator through phosphorylation of SpoOA. Like sensor/kinase proteins, the SpoOB-P intermediate is phosphorylated on a histidine residue (Burbuly et al., 1991).

5. The function of the phosphorelay.

With the onset of starvation, the primary purpose of the phosphorelay is to convert a sporulation signal into activation of SpoOA through protein phosphorylation. It should be recognized that because the same phosphate group is passed from KinA or KinB, through SpoOF and SpoOB, to SpoOA, the phosphorelay forms a linear transduction system and thus does not amplify a sporulation signal. Therefore, the reason for inserting the two phosphotransferase proteins into the phosphorelay is a matter of conjecture. SpoOF and SpoOB may simply be required to funnel sensory input into a single transduction system. This would allow SpoOA to 'communicate' with several different sensor/kinases each monitoring different aspects of the cell's nutritional state. Alternatively, these proteins may provide additional points of control with which to coordinate the activation of SpoOA and, ultimately, induction of sporogenesis, with other cellular processes. It is apparent that nutritional, cell density and cell cycle signals are
all integrated into the phosphorelay circuitry (Grossman et al., 1991; Ireton et al., 1993; Hoch, 1993). The mechanisms that accomplish this are still largely undefined although they presumably effect the flux of phosphate through the pathway.

Recently, it has been reported that Spo0E, Spo0L and Spo0P are phosphatases that antagonize signal transduction through the phosphorelay (Perego and Hoch, 1991; Ohlsen et al., 1994; Perego et al., 1994). In vitro experiments have demonstrated that Spo0A-P and Spo0F-P are targets for Spo0E and Spo0L phosphatase activities, respectively. Because of significant sequence identity between spo0L and spo0P it is expected that Spo0P will also prove to be a phosphatase of Spo0F-P. The induction profiles of spo0E, spo0L and spo0P gene expression suggest that each is controlled by a different regulatory system. Therefore, it has been proposed that the products of these genes may function to prevent sporulation under various physiological conditions by influencing the level of Spo0A phosphorylation (Perego et al., 1994).

6. The regulating properties of Spo0A-P.

In addition to being sporulation defective, cells that carry spo0A mutations fail to become competent, or secrete proteases and antibiotics following entry into stationary phase (Hoch, 1976). Many aspects of the spo0A phenotype can be attributed to the failure to repress abrB whose product functions to block the expression of transition-state genes while cells grow exponentially (Introduction II, 1.a.) (Zuber and Losick, 1987; Perego et al., 1988). However, the repression of abrB can not be the sole function of Spo0A because cells that contain mutations in both abrB and spo0A are still spo-.

These cells fail to induce the early spo genes which genetic experiments have determined are dependent on Spo0A and the other phosphorelay proteins for activation. Thus Spo0A-P was proposed to be an 'ambiaactive' regulator of transcription exerting both negative and positive control over transition-state and early sporulation genes (Perego et al., 1991b).
An *in vitro* examination demonstrated that purified Spo0A repressed transcription from the *abrB* promoter (Strauch *et al.*, 1989; Strauch *et al.*, 1990). Moreover, the protein was shown to bind two DNA sequences, separated by a single helical turn located downstream of the transcription start-site. These sequences, called 0A boxes, defined the canonical Spo0A binding site as 5'-TGNCGAA-3' (Strauch *et al.*, 1990). Examination of DNA sequences of several *spo0* and *spoII* loci believed to be dependent on Spo0A-P for activation, revealed that many of these genes contained putative 0A boxes in proximity to their transcription start-sites. The 0A boxes were found in both orientations and in genes transcribed by either Eσ^A^ or Eσ^H^ forms of RNA polymerase (Spiegelman *et al.*, 1994). Among those included in the Spo0A regulon were *spoIIA*, *spoIIIG* and its own gene, *spo0A*. *spo0A* is transcribed from tandem promoters by Eσ^A^ (Pv) or Eσ^H^ (Ps) (Yamashita *et al.*, 1986; Chibazakura *et al.*, 1991). It is has been shown that Pv produces a low constitutive level of Spo0A protein in exponentially growing cells, while Ps provides a substantial increase in *spo0A* transcription at the onset of sporulation (Strauch *et al.*, 1992). In the event that sporulation signals are detected, it appears that the small amount of Spo0A in vegetative cells becomes phosphorylated. This results in a burst of Spo0A synthesis followed by activation of the Spo0A-P dependent genes (Strauch *et al.*, 1992).

As noted previously, the *spoIIA* and *spoIIIG* operons encode σ^F^ and σ^E^, the first sigma factors that are devoted exclusively to the transcription of developmental genes (Introduction II, 1.b.). Consequently, the activation of these operons represents a significant step toward cell differentiation. The involvement of Spo0A and the other phosphorelay proteins in regulating σ^F^ and σ^E^ synthesis provides potential mechanism for the initiation of sporulation. The phosphorelay could link the sensing of starvation signals to the induction of a sigma factor cascade by activating Spo0A. Thus, the primary objective of the work presented in this thesis was to examine the role of Spo0A(-P) in regulating transcription initiation at the *spoIIA* and *spoIIIG* promoters.
IV. Review of transcription initiation.

1. Promoter structure.

*E. coli* and *B. subtilis* promoters are often characterized by conserved hexameric DNA sequences at the -35 and -10 positions relative to the transcription start-site, and an intervening spacer with an optimal length of 16-17 bps (von Hippel *et al.*, 1984; McClure, 1985; Travers, 1987). The spacer effects the linear and angular separation of the -35 and -10 hexamers on the DNA axis. Occasionally, flanking DNA sequences are important to the transcriptional activity of specific promoters (Ross *et al.*, 1993). Structure/function investigations of several *E. coli* promoters have determined that the -35 and -10 sequences are essential for promoter recognition by RNA polymerase. Therefore, these two elements and the length of the spacer between them, contribute to the transcriptional activity of a given promoter (Helman and Chamberlin, 1988; Stefano and Gralla, 1982b). Generally, there is good correlation between adherence to canonical promoter structure and strong promoter activity in *vitro* (Stefano and Gralla, 1982a; Berg and von Hippel, 1987). Genes and operons that rely on positive regulation for activation have promoters characterized by weak transcriptional activity when studied in *vitro*. These promoters often have unusual sequences and interact poorly with RNA polymerase in the absence of a specific trans-activating factor (Collado-Vidas *et al.*, 1991).

2. The transcription initiation pathway.

It is now generally accepted that promoter specific transcription initiation by RNA polymerase is preceded by a minimum of three reaction steps (for reviews of transcription initiation see Chamberlin, 1974; McClure, 1985; Travers, 1987; Gralla, 1990). The transcription initiation pathway is represented in the following model;

\[
R + P \rightarrow C \rightarrow I \rightarrow O \rightarrow RNA\ synthesis
\]
where \( R \) is the polymerase, \( P \) is the promoter and \( C \), \( I \), and \( O \) represent closed, intermediate and open complexes, respectively.

Transcription initiation begins as a bimolecular reaction where tight binding of the RNA polymerase to promoter DNA results in the formation of a closed complex. It is believed that the polymerase is always in close proximity to the DNA because of its high affinity for non-specific binding sites. Consequently, the enzyme locates promoter sequences by one dimensional diffusion along the surface of the double helix (von Hippel et al., 1984; Mazur and Record, 1989). This allows for rates of association that are often in excess over those predicted for a diffusion limited process in solution (\( 10^8 \text{M}^{-1}\text{sec}^{-1} \)). The extent of RNA polymerase/DNA contact in closed complexes formed at different promoters is variable. Footprinting experiments have generally shown that polymerase covers 50 -70 bps of promoter DNA (Siebenlist et al., 1980; Travers, 1987). Protein/DNA crosslinking studies have demonstrated that the \( \beta \) and \( \beta' \) subunits of the polymerase have multiple contacts with promoter DNA in addition to a tight association between the \( \sigma \) subunit and the -35 and -10 domains (Chenchick et al., 1981).

Evidence for an intermediate step in the initiation pathway has been derived from kinetic, thermodynamic, and footprinting studies of initiation reactions carried out \textit{in vitro} (Buc and McClure, 1985, Spassky et al., 1985; Roe et al. 1985; Cowing et al., 1989; Schickor et al., 1990). The lack of salt dependency and a large negative change in heat capacity associated with the \( C \rightarrow I \) step has been interpreted as evidence for a conformational change within the polymerase (Ha et al., 1989; Leirmo and Record, 1990). It has been postulated that the change in polymerase structure coincides with a nucleation of DNA strand separation initially localized to the -10 region of the promoter (Roe et al. 1985). This is followed by an expansion and subsequent migration of the melted region to encompass the transcription start-site resulting in the formation of an open complex (Leirmo and Record, 1990). Data from experiments which have used reagents that probe open complex structure indicate that 1 to 1.5 turns of the DNA helix
are unwound prior to the initiation of RNA synthesis (Wang et al., 1977; Gamper and Hearst, 1982; Amouyal and Buc, 1987; Buckle and Buc, 1989). Normally, open complexes formed at most E. coli promoters are very stable and initiate RNA synthesis rapidly when provided with nucleoside triphosphates (Spassky et al., 1985; Straney and Crothers, 1985; Levin et al., 1987).

3. Transcription factors and their influence on the initiation reaction.

Transcription factors usually bind to discrete DNA sequences located near the promoters they activate. A survey of positively regulated promoters has established that binding sites are usually near the -40 position and often overlap the -35 polymerase recognition site (Collado-Vidas et al., 1991). Once bound to the DNA, transcription factors influence the rate of transcription initiation in different ways. The regulatory factor can assist in the binding of polymerase to the promoter. This is the case for OmpR which regulates porin synthesis in Gram-negative bacteria, and PhoB, which controls the phosphate uptake regulon. Both OmpR and PhoB compensate for unusual -35 polymerase recognition sequences in their target promoters by facilitating the formation of a closed complex (Makino et al., 1988; Tsung et al., 1990). Alternatively, a transcription factor may catalyze an isomerization step after polymerase has bound to the promoter, as predicted for activation of the \( \lambda P_{RM} \) promoter by the cI protein, or NtrC which controls the glutamine synthetase gene of enteric bacteria. It has been demonstrated that cI stimulates DNA strand separation in a polymerase/\( \lambda P_{RM} \) closed complex resulting in a transcriptionally active open complex (Hawley and McClure, 1982; Li et al., 1994). NtrC displays ATPase activity and catalyzes open complex formation compensating for the inability of the \( \sigma^{54} \) form of RNA polymerase to melt promoter DNA (Kustu, et al., 1989; Popham et al., 1989).
V. The spoIIG and spoI IA promoters.

1. The spoIIG promoter ($P_{spoIIG}$).

The spoIIG operon contains two translational units that are essential to sporulation. The second of these is the structural gene for $\sigma^E$ (Kenney and Moran, 1987). spoIIG transcription begins approximately 60 minutes after the initiation of sporulation (Kenney et al., 1991). An analysis of the DNA sequence upstream from the transcription start-site revealed two domains with high identity to the canonical -35 and -10 recognition sites for a $\sigma^A$-type promoter (Kenney et al., 1989) (Figure 2). Nevertheless, the positioning of these sites did not conform to a conventional $\sigma^A$ promoter because the intervening spacer region was 22 bp long rather than the 16 - 17 bps commonly found for B. subtilis promoters. The increased spacer length would alter the rotational orientation of the -35 and -10 sites drastically. It has been proposed that the unusual structure of $P_{spoIIG}$ would prohibit appreciable transcription during exponential growth and that an ancillary protein must function to activate the spoIIG operon at the onset of sporulation (Kenney et al., 1989).

The interaction of Spo0A and $P_{spoIIG}$ was originally investigated by C. Moran and coworkers. Using electrophoretic mobility shift and DNaseI protection assays, they determined that purified Spo0A protein bound to two regions upstream of $P_{spoIIG}$ (Satola et al., 1991). The DNA sequences within these regions contained OA boxes in the opposite orientation to those found downstream from the abrB promoter. Moreover, point mutations within these sites proved to be deleterious to spoIIG transcription in vivo (Satola et al., 1991). Moran and colleagues tested the effect of Spo0A on spoIIG transcription by adding Spo0A protein to in vitro transcription assays that contained purified E$\sigma^A$ and promoter template (Satola et al., 1992). These experiments demonstrated that non-phosphorylated Spo0A stimulated transcription from $P_{spoIIG}$. As an initial investigation into the effect of phosphorylation on Spo0A activity, acetyl-phosphate was used to obtain a low level of phosphorylated Spo0A. When
The DNA sequence shown corresponds to the nontranscribed strand of *P*<sub>spoIIG</sub> and includes the minimal upstream sequences required for wildtype levels of *spoIIG* expression *in vivo* (Satola et al., 1991). Nucleotide positions indicated below the sequence are relative to the transcription start-site (●). The -35 and -10 RNA polymerase recognition sites are boxed while sequences corresponding to SpoOA binding sites (OA boxes) are underlined. The maximum length of RNA synthesis when ATP and GTP are added to *in vitro* transcription assays as initiating nucleotides (Results I, 2), is designated (●).
phosphorylated protein was added to transcription assays it appeared that stimulation of
spoIIG transcription was enhanced. However, relative levels of \( P_{\text{spoIIG}} \) transcription
were never quantitated (Baldus et al., 1994).

2. The \( \text{spoIIA} \) promoter (\( P_{\text{spoIIA}} \)).

The \( \text{spoIIA} \) operon contains three loci, with the third being the structural gene for \( \sigma^F \)
(Fort and Piggot, 1984; Savva and Mandelstam, 1986). The products of the two
promoter proximal genes act synergistically to regulate and process the \( \sigma^F \) precursor
polypeptide (Schmidt et al., 1990; Challoner-Courtney and Yudkin, 1993; Min et al.,
1993). Expression of the \( \text{spoIIA} \) operon begins approximately 60 minutes after the onset
of sporulation (Savva and Mandelstam, 1986; Errington and Mandelstam, 1986). Wu
and collaborators also demonstrated that \( \text{spoIIA} \) is transcribed by the \( E\sigma^H \) form of RNA
polymerase (Wu et al., 1991).

An initial investigation of the regulation of \( \text{spoIIA} \) expression involved the
construction of a series of deletions that extended into the upstream region of the
promoter and corresponded with a graded reduction in transcriptional activity of the
promoter \textit{in vivo} (Trach et al., 1991). Analysis of DNA sequences revealed several
potential \( 0A \) boxes upstream of the \( \text{spoIIA} \) transcription start-site (Figure 3) and it was
determined through DNaseI footprinting that Spo0A bound to this region of the
promoter (Trach et al., 1991).

VI. Main research objectives.

1. Investigation into the regulation of \( P_{\text{spoIIG}} \) and \( P_{\text{spoIIA}} \).

The central aim of this thesis was to undertake a biochemical analysis of the
transcriptional activation of \( P_{\text{spoIIG}} \) and \( P_{\text{spoIIA}} \) by Spo0A. This was accomplished by
using recombinant KinA, Spo0F, Spo0B and Spo0A proteins to duplicate the
phosphorelay reaction \textit{in vitro} and thereby obtain high levels of phosphorylated Spo0A
Figure 3. The \textit{P\textsubscript{spoIIA}} sequence.

The DNA sequence shown corresponds to the non-transcribed strand of \textit{P\textsubscript{spoIIA}}. Nucleotide positions indicated below the sequence are relative to the transcription start-site (\textbullet\textarrowright) \cite{Wu1991}. Putative Spo0A binding sites and their relative orientations (5'-TGNGCAA-3') are indicated above the sequence (\textbullet\textarrowright). The endpoints for three deletions to upstream regions of the promoter and the \textit{in vivo} transcription frequencies associated with these mutations relative to wildtype promoter constructs, are also shown (Introduction V, 2).
(Spo0A-P). Samples of phosphorelay reactions were added to a transcription assay system to quantitatively assess the effect on $P_{spolIG}$ and $P_{spolIIA}$ activity. These experiments demonstrated that the transcriptional activity of $P_{spolIG}$ and $P_{spolIIA}$ was stimulated by Spo0A-P. This provided biochemical evidence to support the hypothesis that the phosphorelay controls the initiation of a sporulation response by functioning as a transduction system to convert sporulation signals into the activation of Spo0A.

Once it had been established that phosphorylation enhanced the ability of Spo0A to stimulate transcription, the mechanism through which Spo0A-P influenced transcription initiation was examined. This investigation was confined to the activation of $P_{spolIG}$ and involved a kinetic analysis of the effect of Spo0A-P on rates of transcription initiation. These experiments indicated that Spo0A-P accelerated the rate of initiation by effecting an intermediate step in the overall reaction. This interpretation was supported by DNaseI protection assays which demonstrated that Spo0A and Spo0A-P influenced isomerizations in polymerase/$P_{spolIG}$ ternary complexes.
Materials and Methods

I. Standard molecular biology techniques.

1. Plasmid DNA restriction digests.

Except for large scale preparations of \(P_{spolIG}\) template discussed below, reaction volumes were generally 10 - 20 \(\mu\text{L}\) and the plasmid DNA concentration was 50 - 100 nM. Normally, 5 - 10 units of restriction enzyme was added (Bethesda Research Laboratories, New England Biolabs, Pharmacia) to digests that contained buffer provided by the supplier of the enzyme and the reactions were incubated at 37° C for approximately one hour. Reaction samples were usually analyzed following electrophoresis through mini-agarose gels (Materials and Methods I, 4).

2. Ligation reactions and transformation of competent cells.

The subcloning of promoter DNA fragments was accomplished by mixing insert and vector DNAs in reaction volumes that were generally 20 - 50 \(\mu\text{L}\) depending on the total amount of DNA used. Usually, 5 - 10 units of T4 DNA ligase (Bethesda Research Laboratories) was added to each reaction. Ligations involving fragments with compatible ends were incubated at room temperature for 2 - 4 hours, while reactions that contained blunt ended DNA fragments were incubated at 16° C overnight. Ligation reactions were diluted 1 : 5 with TE buffer (10 mM Tris-Cl pH (8.0), 1.0 mM EDTA) prior to being used for the transformation of competent cells. Transformations were carried out using competent cells (DH5α strain; \(hsdR17\) (\(r_k^-, m_k^+\), \(recA1\)) purchased from Bethesda Research Laboratories, using the procedure recommended by the supplier.

3. Plasmid DNA preparations.

Boiling lysis or cleared lysis procedures were used for small or large scale preparations of plasmid DNA, respectively, and were carried out as described by Sambrook et al. (1989). A CsCl (Schwarz/Mann Biotech optical grade) density gradient
procedure, was used to purify large scale plasmid preparations and was followed by several butanol extractions to remove ethidium bromide. The DNA was dialyzed in 3 - 4 exchanges of 4 L of TE buffer (Sambrook et al., 1989). DNA concentration and purity was determined by absorbance readings at 260 and 280 nm. Plasmid DNA was stored in TE buffer at 4°C.

4. Agarose and polyacrylamide gel electrophoresis.

Electrophoresis of DNA, RNA or proteins, was carried out in agarose or polyacrylamide gels as described by Sambrook et al. (1989). Mini-agarose gels (0.7 - 1.4 %) for analysis of DNA restriction digests, were prepared on 5 x 8 cm glass slides and contained 1.5 μg/ml ethidium bromide. DNA was generally electrophoresed in 1/2 X TBE buffer (45 mM Trizma Base, 45 mM boric acid and 1 mM EDTA (pH 8.0)) for 30 to 45 minutes (8 - 10 volts/cm) after which bands of DNA fragments were observed by placing the gels on a UV transilluminator (Ultra-Violet Products Inc.).

Phosphorelay proteins were examined by electrophoresing purified protein, or samples from phosphorelay mixtures, through 15 % SDS - polyacrylamide gels. Gels that contained samples from radioactive phosphorelay reactions used to quantitate the level of Spo0A phosphorylation (Materials and Methods V, 1), were subjected to electrophoresis at 10 - 15 volts/cm. RNA polymerase was examined at various stages of purification after electrophoresis through 14 - 20 % exponential gradient SDS - polyacrylamide gels (Dobinson and Spiegelman, 1987) at 20 - 25 volts/cm. All nonradioactive proteins were observed after staining gels with Coomassie Brilliant Blue R (Sigma Chemical Co.). \(^{32}\)P - labeled protein bands were detected by autoradiography after an overnight exposure to x-ray film (Kodak X-Omat\textsuperscript{TM} RP) at 4 °C.

\(^{32}\)P - labeled RNA from transcription assays was separated by electrophoresis through 7.0 M urea, polyacrylamide gels (6 - 8 %). These gels were electrophoresed in 1/2 X TBE at 40 - 50 volts/cm. RNA bands were detected by autoradiography following an overnight exposure to x-ray film at -70 °C. \(^{32}\)P - labeled \(P_{spoIIIG}\) DNA used for
DNaseI footprinting experiments, was isolated from non-denaturing polyacrylamide gels electrophoresed in 1 X TBE buffer (89 mM Trizma Base, 89 mM boric acid and 2 mM EDTA (pH 8.0)) at 10 - 12 volts/cm. Bands containing $^{32}$P - labeled $P_{spolIG}$ fragments were located by autoradiography, excised from the gel, and electroeluted in dialysis tubing as described by Sambrook et al. (1989).

5. DNA sequencing.

DNA sequencing reactions were carried out using a Sequenase version 2.0 kit purchased from United States Biochemical Corporation and the protocol recommended by the supplier. Double stranded sequencing reactions were carried out using [$\alpha$-$^{32}$P]dATP (3000 Ci/mmol; NEN) and the various primers described in Materials and Methods IV, 7 & VI, 1. The reactions were analyzed following electrophoresis through 7 M urea, polyacrylamide gels (6 - 8%) in 1/2 X TBE.

II. Promoter constructs.

1. Subcloning of $P_{spolIA}$.

Construction of pIIA-28 which carried the $spolIA$ promoter, began with the cloning of a 1138 bp DNA fragment isolated from the vector pKK232-8 (Brosius, 1984) following a PvuII restriction digest. This fragment contained the rho independent tandem terminators from the rRNA $rrnB$ operon of *E. coli*, and was cloned into the HincII site of pUC18. The resulting plasmid was called pPS-28 and could be used to test supercoiled forms of promoter template in transcription assays.

A 950 bp *HindIII* - *Aval* DNA fragment, containing DNA sequences corresponding to the -200 to +700 region of the *spolIIA* operon was isolated from the plasmid pPP115 (Wu and Piggot, unpublished observations) and was subcloned into pPS-28 creating pIIA-28. The structure of pIIA-28 was confirmed by DNA sequencing, which showed that the *spolIIA* promoter was oriented such that transcription was directed toward the $rrnB$ $T_1$+$T_2$ terminators positioned approximately 350 bps downstream.
2. The $P_{spolIG}$ construct.

The plasmid pUCIIGtrpA which carried the $P_{spolIG}$, was a generous gift from C. Moran. This plasmid carried a portion of the spoIIG operon roughly extending from the -100 to the +130 position relative to the transcription start site. This fragment had been cloned into the HindIII - BamHI sites of pUC19 (Satola et al., 1991). The promoter was positioned approximately 160 bp upstream from a rho independent transcription terminator isolated from the trpA gene of E. coli which had been cloned into the HincII site of the vector.

3. The $P_{A2}$ construct.

The plasmid pKKA2 was constructed by subcloning a 200 bp DNA fragment obtained from p328-5 (Dobinson and Spiegelman, 1985) and was ligated into a site upstream from the promoterless chloramphenicol resistance gene of pKK232-8 (Brosius, 1984). This template was linearized by a HindIII restriction digest and used to produce run-off transcription products in transcription assays.

III. Isolation and purification of RNA polymerase, $\sigma^H$, and the phosphorelay proteins.

1. Purification of E$\sigma^A$.

RNA polymerase $\sigma^A$ holoenzyme used for $P_{spolIG}$ and $P_{A2}$ transcription assays, was isolated from B. subtilis strain 168S as described by Dobinson and Spiegelman (1985). This procedure was modified to prepare the holoenzyme used in $P_{spolIIA}$ transcription assays. Following the glycerol gradient step, fractions containing active enzyme were combined, concentrated, and loaded onto a 5 cc heparin - Sepharose column that had been equilibrated with buffer containing 50 mM sodium chloride, 10 mM Tris-Cl (pH 7.9), 10 mM EDTA, 10 mM MgCl$_2$, 10 % glycerol, 10 mM $\beta$-2-mercaptoethanol and 60 $\mu$M phenylmethyl sulfonyl fluoride. The column was washed with 10 mL of the same buffer except that 0.1 M potassium acetate was substituted for sodium chloride. The
polymerase was then eluted with the above buffer supplemented with 1.2 M potassium glutamate. The glycerol content of the polymerase preparation was increased to 50% and the enzyme was stored at -20°C.

2. Preparation of core RNA polymerase.

Core RNA polymerase used for *in vitro* transcription of *P*<sub>sppllA</sub>, was isolated by diluting purified σ<sup>A</sup> RNA polymerase with P cell buffer (0.05 M Hepes pH (7.9), 10 mM EDTA (pH 8.0), 10 mM β-2-mercaptoethanol, 60 μM phenylmethyl sulfonyle fluoride and 20% glycerol). The polymerase was loaded onto a 10 cc phospho-cellulose column that had been equilibrated with P cell buffer containing 0.1 M potassium glutamate. The column was washed with five column volumes of the same buffer and the polymerase was eluted with a 100 mL linear gradient of 0.1 - 1.5 M potassium glutamate in P cell buffer. Core polymerase was observed to elute at potassium glutamate concentrations between 0.30 and 0.45 M. Fractions containing polymerase which had been depleted of the σ<sup>A</sup> subunit, as determined from SDS - PAGE analysis, were pooled and concentrated. Glycerol was added to increase its content to 50%, and the polymerase preparation was stored at -70°C.

3. Purification of σ<sup>H</sup> protein.

Recombinant σ<sup>H</sup> protein used in *sppllA* transcription assays, was provided by J. A. Hoch and colleagues. The method used to purify the protein has been previously reported (Trach *et al.*, 1991).

4. Purification of the phosphorelay proteins.

Recombinant KinA, Spo0F, Spo0B and Spo0A proteins were supplied by the J. A. Hoch laboratory. The procedures used to purify these proteins has been reported elsewhere (Burbulys *et al.*, 1991). The concentrations of various phosphorelay protein preparations was determined from absorbance readings at 280 nM by the Hoch laboratory.
IV. In vitro transcription assay procedures.

1. Preparation of the $P_{spoIIA}$ and $P_A2$ templates.

Because $P_{spoIIA}$ was assayed in supercoiled form, no processing of pIIA-28 was required to prepare the template for transcription assays. For assays that involved $P_A2$, the plasmid pKKA2 was cleaved with HindIII. This digest cleaved the DNA at a single site approximately 170 bp downstream of the transcription start site so that the template produced a runoff product in transcription assays.

2. Preparation and isolation of the $P_{spoIIG}$ template.

$P_{spoIIG}$ was assayed as supercoiled or linearized DNA template. To prepare a large amount of linearized $P_{spoIIG}$ template, approximately 130 - 150 µg of pUCIIIGtrpA was digested with PvuII, which released a promoter fragment approximately 600 bp in length. The entire digestion reaction (300 - 350 µL) was loaded into the wells a 12 x 12 cm agarose gel (1.5 %) and electrophoresed in 1 x TBE buffer (at approximately 8 - 10 volts/cm). Bands containing the promoter fragment were located by placing the gel on a UV transilluminator and were recovered using a DEAE membrane isolation procedure (Sambrook et al., 1989). Once the DNA had been eluted from the membrane, it was butanol extracted twice, diluted to 0.25 M NaCl with sterile distilled water, and precipitated with 3 volumes of 95 % ethanol. The promoter fragment was resuspended in 0.5 mL of DNA storage buffer (10 mM Hepes pH 8.0, 40 mM KAc and 1 mM EDTA), and its concentration was determined by absorption readings at 260 nm. Typically, the promoter concentration was found to be between 80 - 150 nM. Because the promoter fragment also carried the $trpA$ transcription terminator (Materials and Methods II, 2), $P_{spoIIG}$ transcription assays, whether performed on supercoiled plasmid or DNA fragments, produced a transcript approximately 160 bases in length.

3. In vitro transcription assays performed on $P_{spoIIG}$ and $P_A2$.

Transcription assays used to test the effect of Spo0A(-P) on $P_{spoIIG}$ and $P_A2$ transcriptional activity, were conducted in a total volume of 20 µL and a DNA
concentration that was normally 2-5nM. The assays were carried out in 0.65 mL microfuge tubes by mixing template DNA with 1X transcription buffer (40 mM Hepes (pH 8.0), 5 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM dithiothreitol and 0.1 mg/mL bovine serum albumin) to a volume of 14 μL. These mixtures also contained 0.4 μM ATP, 0.01 μM GTP and 2-5 μCi of [α-32P]-GTP (800 Ci/mmol; NEN). The tubes were warmed to the desired assay temperature (usually 37°C) for 2 - 4 minutes before adding 2 μL of a phosphorelay sample diluted to an appropriate concentration (Materials and Methods V, 1). Spo0A(-P) and DNA mixtures were incubated 4 - 6 minutes before an initiation reaction was begun with 2 μL of purified σ7A. Reaction tubes were incubated an additional 4 - 6 minutes before adding 2 μL of a mixture containing 1.0 mg/ml heparin, 4.0 mM CTP and 4.0 mM UTP. The heparin/nucleotide mixture effectively stopped initiation reactions but permitted RNA elongation from heparin-resistant complexes (Results, section I, 2). After 10 minutes, transcript elongation was assumed to be complete. Five μL of denaturing gel loading buffer (8.0 M urea, 180 mM Trizma base, 180 mM boric acid, 0.004 mM EDTA, 0.02 % bromophenol blue, 0.02 % xylene cyanol) was added to the reactions prior to electrophoresis through 8 % denaturing polyacrylamide gels. Transcript bands were detected by autoradiography and were excised from the gels. The level of Cerenkov radiation in the gel slices containing transcripts was measured by scintillation counting and was used to determine promoter activity (Materials and Methods IV, 5).

4. \( P_{spoIIIC} \) transcription rate assays.

Transcription rate assays were performed on purified DNA fragments that carried \( P_{spoIIIC} \). The final DNA concentration was usually 2 nM. The assays were begun by mixing the DNA, 0.4 μM ATP, 0.01 μM GTP and 15 - 20 μCi of [α-32P]GTP (800 Ci/mmol; NEN) in 1X transcription buffer to a volume of 112 μL. Rate assays that examined the effect of salt concentration on transcription initiation rates were conducted by adding the desired amount of NaCl, KCl, or KAc to this mixture. Once the mixture
had been warmed to 37°C, 16 μL sample that contained an appropriate dilution of an equilibrated phosphorelay mixture (Materials and Methods IV, 5), was added and incubated with the DNA for 3 minutes. An initiation time-course reaction was begun with the addition of 18 μL of RNA polymerase (EσA) that had been previously diluted to obtain the desired concentration of active enzyme. At regular intervals, 18 μL samples were removed and added to 0.65 mL microfuge tubes containing 2 μL of 1.0 mg/mL heparin, 4 μM CTP and 4 μM UTP. This mixture blocked further transcription initiation but allowed transcript elongation from heparin-resistant complexes. Transcript elongation reactions were stopped with 5 μL of denaturing gel loading buffer and the reactions were electrophoresed through 8% SDS-polyacrylamide gels. The extent of transcription in each sample was quantitated as described in Materials and Methods IV, 5.

5. Determination of PspoIIG transcription activity.

The number of full length transcripts produced in PspoIIG transcription assays was calculated by measuring the level of Cerenkov radiation in a 2 μL sample of labeling mix (containing [α-32P]GTP/GTP) that had been added to transcription assays. This provided a measure of the radioactivity (cpm) added to each transcription assay. This value was divided by the total number of GTP molecules in transcription reactions, and multiplied by 36, the number of GTPs incorporated into a full length PspoIIG transcript. The final value was taken as a measure of cpm per PspoIIG transcript and was used to quantitate the number of transcripts produced by comparison to the level of Cerenkov radiation in gel slices containing PspoIIG products.

6. PspoIIA transcription assays.

Transcription assays conducted on PspoIIA were initiated by adding 2 μL of RNA polymerase that consisted of recombinant σH protein mixed with either core polymerase or EσA, to a 14 μL mixture of PspoIIA template and buffer that contained 40 mM Hepes pH 8.0, 10 mM MgCl₂, 0.1 mM dithiothreitol, 0.1 mM EDTA and 100 μg/mL bovine
serum albumin. The polymerase and DNA were incubated together for 5 minutes, after which the reactions were subjected to a 5 minute challenge with 2 μL of 1.0 mg/mL heparin. Transcript elongation from heparin-resistant complexes were initiated with the addition of GTP, ATP and CTP to 0.4μM, UTP to 0.01μM and 2-4 μCi of [α-32P]UTP (800 Ci/mmol, Amersham). Elongation reactions were incubated for 10 minutes and then terminated with 5 μL of denaturing gel loading buffer. The reactions were electrophoresed through 5 % urea - polyacrylamide gels. 32P-labeled RNA was detected by autoradiography and quantitated by measuring Cerenkov radiation levels in gel slices.

7. Primer extension analysis of P_spoIIG and P_spoIIA transcripts.

To determine the start site for in vitro transcription from P_spoIIG and P_spoIIA promoters, RNA from transcription assays was recovered from polyacrylamide gels by electroelution in dialysis tubing (Sambrook et al., 1989). The transcripts were ethanol precipitated, resuspended in sterile distilled water and used as template for primer extension reactions.

Primer extension reactions were carried out using purchased DNA primers that were complementary to sequences downstream from either P_spoIIA or P_spoIIG (spoIIA, 5'-GGCGAATATCATCCTTCTCC-3'; spoIIG, 5'-TCAGAAAATAAATGCCG-Y). The primers were 5' end labeled by mixing 1 μL of primer (40 OD_{260}/mL), approximately 300 μCi of [γ-32P]ATP (8000 Ci/mmol; ICN), and 10 units of T4 polynucleotide kinase (Bethesda Research Laboratories) in 20 μL of 1 X kinase reaction buffer (50 mM Tris-Cl (pH 7.6), 10 mM MgCl₂, 5 mM dithiothreitol, 0.1 mM spermidine, 0.1 mM EDTA (pH 8.0)). Labeling reactions were incubated at 37° C for 45 minutes and then at 65° C for 10 minutes to inactivate the enzyme. 32P - labeled primer was separated from unincorporated nucleotide by NAC columns using the procedure recommended by the supplier (Bethesda Research Laboratories). The efficiency of end labeling was determined by measuring the level of Cerenkov radiation in a diluted sample of eluted primer.
Primer extension reactions were begun by mixing RNA template with approximately 50,000 cpm of the appropriate end labeled primer in 1 X annealing buffer (50 mM Tris-Cl (pH 8.0), 60 mM NaCl, 0.5 mM dithiothreitol and 1 mM EDTA) to a volume of 5 \mu L. The primer and template were incubated at 90°C for 2 minutes. Annealing reactions were allowed to cool slowly to 45°C before adding 2 \mu L of 5 X focus buffer (250 mM Tris-Cl (pH 8.3), 375 mM KCl, 50 mM dithiothreitol, 15 mM MgCl₂), 2 \mu L of a mixture of dATP, dCTP, dTTP and dGTP (2.5 mM each), 0.5 \mu L sterile distilled water and 0.5 \mu L of avian myeloblastosis virus reverse transcriptase (Promega). The reaction was incubated at 45°C for 1 hour after which the extension products were ethanol precipitated and resuspended in 5 \mu L of formamide gel loading buffer (2.5 X TBE, 95 % formamide, 0.02 % bromophenyl blue and 0.02 % xylene cyanole). A sample was loaded onto a 7 M urea, 8 % polyacrylamide gel and electrophoresed next to sequencing reactions carried out on promoter DNA with the same primer as used in the extension reaction.

V. Phosphorelay reactions.

1. Typical phosphorelay reaction procedures and quantitation of Spo0A phosphorylation.

Phosphorelay reactions were carried out by mixing recombinant KinA, Spo0F, Spo0B and Spo0A proteins in 1 X transcription buffer. Reaction volumes varied according to the requirements of different experiments, but were usually 20 - 60 \mu L. Normally, Spo0A concentration was 4.0 \mu M, while KinA, Spo0F and Spo0B were approximately 0.4 \mu M each. Kinase activity was initiated with the addition ATP to a final concentration of 1.0 mM and the reactions were incubated for 60 - 90 minutes at room temperature to allow equilibration of the phosphorelay. Samples from equilibrated phosphorelay reactions were added directly to transcription assays following dilution to an appropriate Spo0A(-P) concentration with 1 X transcription buffer.
The degree of Spo0A phosphorylation was quantitated by adding [$\gamma^{32}$P]ATP (8000 Ci/mmol; ICN) to phosphorelay incubations. Once the reaction had equilibrated, samples containing a known amount of Spo0A protein were separated by SDS-PAGE (Materials and Methods I, 4) and protein bands were detected by autoradiography. The level of Cerenkov radiation in gel slices containing Spo0A protein was measured by scintillation counting and was compared to the quantity of radioactivity originally added to the phosphorelay reaction. Normally, 60 - 80 % of the Spo0A protein added to a phosphorelay mixture was phosphorylated once the reaction had equilibrated.

Mixtures of phosphorelay reactions, which were added to transcription assays to assess the effect of nonphosphorylated Spo0A, contained all the phosphorelay reaction components except KinA and ATP. It was necessary to omit KinA from these mixtures to avoid Spo0A phosphorylation once samples were added to transcription assays where ATP was required to form heparin-resistant complexes (Results I, 2). It had been observed that KinA had no direct influence on the level of transcription from either $P_{spoIIG}$ or $P_{spoIIA}$ (Results II, 2). Assays that tested $P_{spoIIG}$ transcription in the absence of Spo0A received either an aliquot of 1 X transcription buffer or a sample from a mixture that included all the phosphorelay proteins except Spo0A.

2. Phosphorelay time-course reactions.

To couple a time-course phosphorelay reaction to $P_{spoIIG}$ transcription assays, two separate kinase reactions were conducted by mixing Spo0A, Spo0F and Spo0B to a concentration of 1.0 μM, and KinA to a concentration of 0.1 μM. At time zero, ATP was added to one reaction to give a final concentration of 50 μM. At various intervals 2.0 μL samples were removed from this reaction and added directly to transcription assays. The second kinase reaction was initiated with an ATP mixture that included approximately 100 μCi of [$\gamma^{32}$P]ATP (8000 Ci/mmol; ICN). The total ATP concentration in the radioactive reaction was also 50 μM. At various times samples were removed from the labeled reaction and mixed with protein gel loading buffer (50
mM Tris-Cl (pH 6.8), 100 mM dithiothreitol, 2 % SDS, 0.02 % bromophenol blue and 10 % glycerol) to arrest kinase activity. The proteins in these samples were later separated on a 15 % SDS-polyacrylamide gel. The level of Spo0A phosphorylation was calculated following a measurement of the Cerenkov radiation in gel slices containing labeled Spo0A protein as described above (Materials and Methods V, 1).

VI. DNaseI protection assays.

1. End labeling of \( P_{spoIIG} \) DNA fragments.

Labeling of the 5' ends of \( P_{spoIIG} \) DNA fragments was carried out by digesting approximately 20 -25 μg of pUCIIGtrpA with either HindIII (to label the transcribed strand) or BamHI (to label the non-transcribed strand). The ends of digested DNA were dephosphorylated with alkaline calf intestinal phosphorylase (Pharmacia). The reactions were then phenol extracted and ethanol precipitated. The DNA was resuspended in 15 μL of sterile distilled water and mixed with 2 μL 10 X kinase buffer, 2 μL \([γ-^{32}P]ATP\) (8000 Ci/mmol; ICN), and 1 μL of T4 polynucleotide kinase (Bethesda Research Laboratories). The kinase reaction was incubated at 37° C for 30 minutes before the enzyme was inactivated by a 15 minute incubation at 65° C. The reaction volume was increased to 50 μL with sterile distilled water before adding 4 μL of \( PvuII \) and 6 μL of the appropriate 10 X restriction buffer to release the labeled fragment from the vector. After a 1 hour digestion, the reaction was loaded onto a 5 % polyacrylamide gel and electrophoresed in 1 X TBE buffer. The \( ^{32}P \) - labeled promoter fragment was localized by autoradiography and electroeluted from gel slices in dialysis tubing (Sambrook et al., 1989). Recovered DNA was ethanol precipitated and resuspended in 100 - 200 μL of DNA storage buffer (10 mM Hepes (pH 8.0), 40 mM KAc and 1 mM EDTA). \( ^{32}P \) labeling of the promoter fragment was quantitated by measuring Cerenkov radiation in a 2 μL sample of the resuspended DNA.
2. DNaseI footprinting of various Spo0A(-P) concentrations.

Individual footprint reactions were carried out by adding 8 - 10 x 10^4 cpm of end labeled $P_{spolIG}$ fragment to 1.7 mL microfuge tubes that contained 0.2 mM ATP in 1 X transcription buffer (Materials and Methods IV, 3). The volume of these mixtures was 16 µL if RNA polymerase was to be added, or 18 µL for Spo0A(-P) footprinting in the absence of polymerase. Reaction tubes were warmed to the desired assay temperature for 2 - 5 minutes before adding 2 µL of phosphorelay reaction sample containing either no Spo0A, Spo0A or Spo0A-P (Materials and Methods V, 1). The protein and DNA were incubated for 5 minutes followed by a second 5 minute incubation after the addition of a 2 µL sample of RNA polymerase. A partial DNA digestion was carried out by adding 4 µL of 4.0 µg/mL DNaseI. The DNA was digested for 10 seconds before the reactions were quenched with 75 µL of DNaseI stop buffer (Hepes (pH 8.0), 0.1 % SDS, 4 mM EDTA, 270 mM NaCl and 3 µg of sonicated calf thymus DNA). The reactions were ethanol precipitated, resuspended in 5 - 8 µL of formamide gel loading buffer and the level of Cerenkov radiation recovered in each sample was determined by scintillation counting. Equal quantities of radioactivity from each footprint reaction were separated on a 8 % urea - polyacrylamide gel. The gel was dried and the DNaseI protection patterns were analyzed following autoradiography.

3. Time-course DNaseI protection assays.

Time-course (or kinetic) footprint assays were carried out by incubating 1.7 mL microfuge tubes to the desired reaction temperature. Each tube contained a 112 µL mixture of approximately 8 x 10^5 cpm of end labeled $P_{spolIG}$ fragment and 0.2 mM ATP, in 1 X transcription buffer. A 16 µL sample was removed and treated as described below to provide a control DNaseI digestion pattern. A 14 µL sample of a phosphorelay mixture containing no Spo0A, Spo0A or Spo0A-P (Materials and Methods V, 1) was added to the remaining DNA and the reactions were incubated for 1 minute. An 18 µL sample was then removed and treated with DNaseI to serve as a control reaction that
provided a DNaseI pattern resulting from the binding of Spo0A or Spo0A-P, in the absence of RNA polymerase. A 12 µL aliquot of RNA polymerase was then added to the reaction to begin a time-course binding reaction where samples were removed at regular intervals and treated with DNaseI.

DNaseI digestions were conducted by adding samples removed from binding reactions to microfuge tubes containing 4 µL of 4 µg/mL DNaseI. In the case of control digestions where DNA samples were 16 or 18 µL, 4 or 2 µL of 1 X transcription buffer was added to the DNaseI solution so that all digestions were carried out in a total volume of 24 µL. The samples were digested for 10 seconds before the addition of 75 µL of DNaseI stop buffer. The footprint reactions were then precipitated, subjected to electrophoresis, and analyzed exactly as described above.

VII. Mutagenesis and end labeling of P_{spoIIG} by polymerase chain reaction.

1. Generation of P_{spoIIG}^{M94/84} by PCR.

To construct the mutant promoter P_{spoIIG}^{M94/84}, a primer was purchased which contained a DNA sequence complementary to the -91 to -71 region of the transcribed strand of P_{spoIIG}. This primer carried two mismatched G residues corresponding to positions -82 and -81 of the 0A box centered at -84 (see Figure 2). A polymerase chain reaction (PCR) utilizing this primer and the universal reverse primer complementary to the vector portion of pUCIIGtrpA, was expected to produce a double stranded P_{spoIIG} construct where a portion of the 0A box at -94 would be deleted in addition to insertion of the two point mutations into the 0A box at -84.

PCR reactions contained 25 ng of pUCIIGtrpA plasmid previously digested with PvuII, the four dNTPs (Pharmacia Ultrapure deoxynucleoside 5'-triphosphates) at 2.5 mM each, 1 pmole each of the mutagenesis primer and universal reverse primer, and 1 µL of Taq polymerase (Boeringer Mannheim) in a final volume of 50 µL. Reactions were carried out in buffer provided by the supplier of Taq polymerase enzyme and were
overlaid with mineral oil. The mutant promoter fragment was amplified by 30 cycles of
denaturation, annealing and extension steps carried out at 95°C for 30 seconds, 54°C
for 1 minute and 72°C for 1 minute, respectively. This was followed by a final DNA
extension reaction at 72°C for 5 minutes. Synthesized DNA fragments were recovered
from 1.4 % agarose gels using the DEAE membrane procedure (Sambrook et al., 1989)
and following ethanol precipitation the DNA was resuspended in DNA storage buffer
and quantitated by absorption readings at 260 nm. Sequencing of the double stranded
DNA product confirmed that two mismatches had been incorporated at the -82 and -81
positions of the OA box at -84, and that the distal half of the OA box centered at -94 had
been deleted.

2. \(^{32}\)P - labeling of \(P_{\text{spoIIG}}^{M94/84}\) for DNaseI protection experiments.

End labeling of \(P_{\text{spoIIG}}^{M94/84}\) promoter fragment for DNaseI protection studies was
accomplished by conducting PCR reactions with the same \(^{32}\)P - labeled primer as used
for primer extension of \(P_{\text{spoIIG}}\) transcripts (Materials and Methods IV, 7). The
\(P_{\text{spoIIG}}^{M94/84}\) promoter fragment was mixed with the mutagenesis primer (Materials and
Methods VI, 1) and the labeled primer and PCR reactions were carried out exactly as
described above. The labeled promoter fragment was electroeluted from a
polyacrylamide gel as described for \(^{32}\)P - labeled wildtype promoter (Materials and
Methods VI, 1). The DNA was ethanol precipitated, resuspended in DNA storage
buffer and stored at 4°C. The labeled fragment was used in DNaseI footprinting
experiments as described for the wild-type promoter (Materials and Methods VI, 2).
Results

I. In vitro $P_{\text{spoII}G}$ transcription assays.

1. Transcription assay procedure.

Transcription assays were initiated by incubating RNA polymerase (Figure 4) with promoter DNA, usually under varying conditions of ionic strength, temperature, or reactant concentrations. Products of these binding reactions were then challenged with heparin, a competitive inhibitor of transcription. Heparin has been shown to bind free polymerase irreversibly and to cause the rapid dissociation of polymerase bound to non-specific sites on the DNA (Walter et al., 1967). Only polymerase that is specifically bound to promoter DNA sequences is resistant to heparin inhibition. Elongation of RNA from heparin-resistant polymerase/promoter complexes was initiated with the addition of nucleoside triphosphates (NTPs). The number of transcripts produced in transcription assays was measured following gel electrophoresis of reaction mixtures (Methods and Materials IV, 5).

Because it rapidly sequesters all polymerase which has not formed resistant complexes, heparin ensures that only a single round of transcription can occur at each promoter when added either before or simultaneously with NTPs. Consequently, transcript production is proportional to the number of heparin-resistant complexes formed in a binding reaction. A measure of the number of transcripts synthesized in a transcription assay provides a quantitative appraisal of promoter activity in terms of heparin-resistant complex formation. In this work, in vitro promoter activity is expressed as the percentage of promoter templates that produced an RNA.

2. Heparin-resistant complex formation at $P_{\text{spoII}G}$.

$P_{\text{spoII}G}$ transcription assays produced no significant transcription when all four NTPs were added to binding reactions simultaneously with heparin. However, when both ATP and GTP were included in polymerase/$P_{\text{spoII}G}$ incubations before the addition of
Figure 4. SDS-polyacrylamide gel of RNA polymerase.

To estimate the relative purity of RNA polymerase during large scale preparations of the enzyme, samples from glycerol gradient fractions (lanes 1 - 10) containing RNA polymerase were separated by a 14 - 20 % exponential gradient SDS-PAGE and stained with Coomassie Blue. (Materials and Methods III, 1). In this example, fractions represented by lanes 1-7 were combined and concentrated by heparin-Sepharose chromatography. Samples of concentrated polymerase were electrophoresed in lanes A and B. Bands representing the largest subunits of RNA polymerase holoenzyme are indicated to the right.
heparin, CTP, and UTP, promoter activity was substantial. Because ATP and GTP are
the first NTPs incorporated into a nascent transcript, this precondition suggested that
only RNA polymerase which had initiated transcription at \( P_{\text{spoIIG}} \) was resistant to
heparin inhibition. Initiating nucleotides are a common requirement for the formation of
heparin-resistant complexes at several \( B.\ subtilis \) promoters when assayed \textit{in vitro}
(Dobinson \textit{et al.}, 1985 & 1987; Whipple and Sonenshein, 1992). In the case of \( P_{\text{spoIIG}} \),
RNA polymerase can synthesize a pppApA dimer when provided with ATP, or a
transcript up to 11 nucleotides in length when supplied with both ATP and GTP (see
Figure 2). The exact step at which initiated complexes became heparin-resistant was
never investigated. However, it appeared that the polymerase must have proceeded past
the synthesis of a dinucleotide since the addition of ATP alone did not yield heparin-
resistant complexes. Heparin-resistant complexes formed at \( P_{\text{spoIIG}} \) were very stable
over time and readily elongated full length transcripts when provided with CTP and
UTP.

3. Mapping of the \( P_{\text{spoIIG}} \) transcription start-site.

To ensure promoter specificity and define the start-site for \textit{in vitro} transcription from
\( P_{\text{spoIIG}} \), RNA produced in transcription assays was electroeluted from polyacrylamide
gel slices and used as template for primer extension analysis (Materials and Methods IV,
7). When compared to \( P_{\text{spoIIG}} \) DNA sequencing reactions (Figure 5), the primer
extension product was found to originate from the same transcription start-site as had
been identified for \( spoIIG \) mRNA produced \textit{in vivo} (Kenney \textit{et al.}, 1989).

II. Effect of Spo0A-P on the transcriptional activity of \( P_{\text{spoIIG}} \).

1. \textit{In vitro} phosphorelay reactions.

To phosphorylate Spo0A, \textit{in vitro} kinase reactions were conducted using
recombinant phosphorelay proteins, KinA, Spo0F, Spo0B, and Spo0A (Figure 6A).
Phosphorelay reactions were incubated at room temperature and were initiated by
Figure 5. Primer extension analysis of RNA produced in $P_{spolIG}$ transcription assays.

RNA from $P_{spolIG}$ transcription assays was isolated from polyacrylamide gel slices by electroelution and was used as template for primer extension reactions (Materials and Methods IV, 7). The extension product was analyzed by PAGE and compared to DNA sequencing reactions using $P_{spolIG}$ template and the same primer as used in the primer extension reaction. The sequence ladder shown corresponds to the non-transcribed strand of $P_{spolIG}$. The -10 RNA polymerase recognition site is indicated to the left. The primer extension product, indicated by the arrow, maps to the position proposed to be the in vivo transcription start-site (Kenney et al., 1989).
adding ATP to a mixture of the four proteins (Materials and Methods V, 1). Labeling of phosphorelay proteins with \([\gamma-^{32}\text{P}]\text{ATP}\), is demonstrated in Figure 6B. Once the phosphorelay reactions were completed and the components had been separated by SDS-PAGE, the degree of Spo0A phosphorylation was determined by measuring Cerenkov radiation in gel slices containing a known amount of Spo0A protein. Normally, it was found that more than 50% of the Spo0A proteins in equilibrated phosphorelay reactions were phosphorylated.

2. Test of phosphorelay components in \(P_{spoII}G\) transcription assays.

To test the effect of Spo0A-P on \(P_{spoII}G\) activity, aliquots taken from equilibrated phosphorelay reactions were added to transcription assays that contained purified DNA fragments that carried \(P_{spoII}G\) (Materials and Methods IV, 2). Because these samples also contained ATP and the other phosphorelay proteins, it was important to verify that none of the phosphorelay components other than Spo0A, affected \(P_{spoII}G\) activity. Figure 7 shows an autoradiogram of labeled RNA products from transcription assays that contained various combinations of phosphorelay components. The transcription assay that contained a sample from an equilibrated phosphorelay reaction (lane 6) exhibited increased RNA production indicating that transcription had been stimulated by Spo0A-P. In contrast, no other assay showed an increase in transcription. Therefore, it was apparent that none of the other kinase reaction components stimulated promoter activity above the basal level of transcription (lane 1). Because the various combinations of phosphorelay proteins were incubated with ATP before they were added to transcription assays, it was concluded that KinA-P, Spo0F-P, and Spo0B-P had no effect on \(P_{spoII}G\) transcription.

3. Effect of Spo0A(-P) concentration on \(P_{spoII}G\) activity.

The effect of phosphorylated or non-phosphorylated Spo0A (Spo0A(-P)) on \(P_{spoII}G\) activity, was investigated by adding serial dilutions of phosphorelay reactions to transcription assays. These samples were incubated with \(P_{spoII}G\) template prior to the
Figure 6A. SDS - polyacrylamide gel containing purified phosphorelay proteins.

Samples of the recombinant proteins used in phosphorelay reactions were electrophoresed through a 15 % SDS - PAGE and stained with Coomassie Blue. Each lane contains: lane 1, KinA (55 kDa); lane 2, SpoOB (28 kDa); lane 3, Spo0F (15 kDa); lane 4, Spo0A<sup>BD</sup> (17 kDa); and lane 5, Spo0A (30 kDa). Spo0A<sup>BD</sup> is a proteolytic product of a trypsin digest of purified Spo0A. It represents the C - terminal half of the intact protein and includes the DNA binding domain. This protein was not added to phosphorelay reactions since it lacks the phospho-acceptor site (Asp<sup>56</sup>), but was added to transcription assays to test its effect on <i>P<sub>spolIG</sub></i> activity (Results IV, 9 & 10).

Figure 6B. Autoradiogram of <sup>32</sup>P - labeled phosphorelay proteins separated by SDS - PAGE.

Samples removed from various mixtures of phosphorelay proteins that had been incubated with [γ<sup>32</sup>P]ATP were separated on a 15 % SDS - polyacrylamide gel. <sup>32</sup>P - labeled proteins were detected by autoradiography (Materials and Methods V, 1). This autoradiogram demonstrates the autophosphorylation of KinA and <sup>32</sup>P - labeling of the other proteins in each reaction. Reactions loaded in each lane contained the following proteins; lane 1, KinA only; lane 2, KinA and Spo0F; lane 3, KinA, Spo0F and Spo0B; lane 4, KinA, Spo0F, Spo0B and Spo0A.
Figure 7. Phosphorelay component test on $P_{\text{spoII}G}$ activity.

Various mixtures of phosphorelay proteins were incubated with ATP and then added to transcription assays that contained $P_{\text{spoII}G}$ template. $^{32}$P-labeled transcripts were separated by electrophoresis through a 6% SDS-polyacrylamide gel and were detected by autoradiography. Lanes 1 - 6 show RNA produced in transcription assays that contained the mixtures of phosphorelay proteins indicated below each lane.
addition of RNA polymerase. Kinase reaction samples that contained non-phosphorylated Spo0A were taken from a mixture of phosphorelay components that did not include either ATP or KinA. It was necessary to omit KinA to avoid phosphorylation of Spo0A once the sample was added to transcription assays which contained ATP (Materials and Methods V, 1). Because there is no independent assay to determine the specific activity of purified recombinant Spo0A, the concentrations of Spo0A added to transcription assays are given as total protein.

Figure 8 shows an autoradiogram of labeled RNA from assays where supercoiled plasmid or purified DNA fragments were used as $P_{spoIIG}$ template. It was observed that $P_{spoIIG}$ transcription from either form of template was stimulated by Spo0A(-P). $P_{spoIIG}$ stimulation reached a maximum when Spo0A-P concentration was 160 nM (Figure 9). This maximum was approximately ten fold higher than the level of transcription measured in assays that did not contain a phosphorelay sample. Addition of non-phosphorylated Spo0A also stimulated $P_{spoIIG}$ transcription, however, the increase was slight and occurred only at the highest Spo0A concentrations.

Because there was no apparent difference in the relative levels of transcription stimulation from supercoiled or relaxed $P_{spoIIG}$ template, it was decided that further investigations into $P_{spoIIG}$ activation would be carried out with linear DNA templates. These assays produced lower levels of background transcription which significantly improved the accuracy of promoter activity measurements.

4. The effect of Spo0A-P on $P_{A2}$ activity.

To determine whether transcription activation by Spo0A-P was promoter specific, phosphorelay samples were added to transcription assays that contained $P_{A2}$, isolated from the Bacillus subtilis bacteriophage, φ29 (Dobinson and Spiegelman, 1985). An examination of the DNA sequences in the vicinity of $P_{A2}$ revealed no obvious 0A boxes. Therefore, Spo0A-P was not expected to affect $P_{A2}$ transcription. As for $P_{spoIIG}$, RNA polymerase requires ATP and GTP as initiating NTPs to form heparin-resistant...
Figure 8. Effect of Spo0A(-P) concentration on \textit{in vitro} transcription from $P_{spoIIG}$.

Samples from serial dilutions of phosphorelay reactions that contained non-phosphorylated Spo0A or Spo0A-P (Materials and Methods V, 4), were added to transcription assays carried out with supercoiled (A) or linear (B) $P_{spoIIG}$. The $^{32}$P labeled transcripts were separated by electrophoresis through a 6% urea-polyacrylamide gel and were detected by autoradiography. The final concentration of Spo0A protein added to each assay was: lane 1, no protein; lane 2, 10 nM; lane 3, 20 nM; lane 4, 40 nM; lane 5, 80 nM; lane 6, 160 nM and lane 7, 320 nM.
Figure 9. Effect of Spo0A(-P) concentration on $P_{spt0IIG}$ activity.

Bands containing the $^{32}$P-labeled $P_{spt0IIG}$ transcripts from transcription assays that contained serial dilutions of nonphosphorylated Spo0A (---) or Spo0A-P (---) (described in Figure 8), were excised from polyacrylamide gels. The number of transcripts was quantitated by measuring Cerenkov radiation (Materials and Methods IV, 5). The number of transcripts divided by the number of $P_{spt0IIG}$ templates in each assay provided a measure of promoter activity and was plotted as a function of total Spo0A protein concentration in the transcription assay. Transcription assays were carried out with supercoiled (A) or linearized (B) $P_{spt0IIG}$ template.
A

![Graph A](image)

B

![Graph B](image)
complexes at $P_{A2}$ in vitro (Dobinson and Spiegelman, 1987). This indicated that RNA polymerase has similar transcription initiation properties at the two promoters. $P_{A2}$ transcription assays were carried out at various concentrations of Spo0A or Spo0A-P as described for assays carried out with $P_{spolIG}$, but no influence on promoter was observed (Figure 10). Thus, it was concluded that stimulation of $P_{spolIG}$ transcriptional activity was a promoter specific effect and probably required binding of Spo0A-P to specific binding sites on the DNA.

5. Coupling of a phosphorelay time-course reaction to $P_{spolIG}$ transcription assays.

To correlate $P_{spolIG}$ transcription stimulation with the degree of Spo0A phosphorylation, a series of transcription assays was coupled to a phosphorelay time-course reaction. Trial time-course experiments had demonstrated that the time required for phosphorelay reactions to equilibrate could be controlled by adjusting ATP concentration. Phosphorelay reactions that contained 50 μM ATP required at least 60 minutes to reach maximum Spo0A phosphorylation. Although this maximum was approximately 50% lower than in reactions that contained higher ATP concentrations, a slower kinase reaction was necessary to carry out consecutive transcription assays that contained phosphorelay samples with intermediate levels of Spo0A phosphorylation.

Spo0A phosphorylation was monitored in a second kinase reaction that contained $[\gamma^{32}P]ATP$. Samples were removed from the radioactive reaction and added to SDS-protein gel loading buffer to denature the proteins thereby terminating kinase activity. The $^{32}P$-labeled proteins were separated by SDS-PAGE and the level of Spo0A phosphorylation was determined for each sample (Materials and Methods V, 1). Labeled and non-labeled phosphorelay reactions were initiated simultaneously to a final ATP concentration of 50 μM. Because they were carried out under nearly identical conditions, it was assumed that the degree of Spo0A phosphorylation in the two reactions was similar.
Figure 10. Effect of increased Spo0A(-P) concentration on \textit{in vitro} transcription from \( P_{A2} \).

Samples from serial dilutions of phosphorelay reactions that contained Spo0A (\( \triangle \)) or Spo0A-P (\( \blacksquare \)) (Materials and Methods V, 1), were added to transcription assays carried out on the \( \phi 29 \) promoter, \( P_{A2} \). \( P_{A2} \) transcriptional activity was quantitated as for the \( P_{spoIIG} \) transcription assays described in Figure 9 and is plotted as a function of total Spo0A protein concentration.
Once kinase activity had been initiated, samples were removed from the non-radioactive reaction at regular intervals and immediately added to \( P_{spoIIG} \) transcription assays to a final Spo0A concentration of 100 nM. Figure 11 shows autoradiograms of \( ^{32} \text{P} \)-labeled proteins from the radioactive phosphorelay reaction, and \( P_{spoIIG} \) transcripts produced in transcription assays that contained non-radioactive kinase reaction samples. It was apparent that \( P_{spoIIG} \) transcription increased dramatically as the phosphorelay progressed (Figure 11B). Spo0A phosphorylation measured in samples removed from the labeled phosphorelay reaction, is plotted in Figure 11C along with the levels of \( P_{spoIIG} \) transcription. This plot demonstrates that increased stimulation of \( P_{spoIIG} \) transcription paralleled Spo0A phosphorylation. The first sample removed from the kinase reaction after it had been initiated with ATP appeared to stimulate \( P_{spoIIG} \) transcription disproportionately. This was probably because of a rapid phosphorylation of Spo0A protein after the kinase sample was added to the transcription assay that contained 400 \( \mu \text{M} \) ATP (Materials and Methods V, 2). Maximum \( P_{spoIIG} \) transcription, obtained with a phosphorelay sample taken 40 minutes after beginning the kinase reaction, was approximately 10 fold higher than in an assay that contained a phosphorelay sample taken prior to initiating kinase activity. This experiment was taken as compelling evidence that Spo0A-P stimulated \( P_{spoIIG} \) activity and that the level of stimulation was dependent on the degree of Spo0A phosphorylation.

III. Investigation of reaction conditions affecting \( P_{spoIIG} \) transcription.

1. Temperature effects on \( P_{spoIIG} \) transcription.

\textit{In vitro} transcription, particularly from relaxed DNA template, has been shown to be a temperature sensitive process (Travers, 1987; Leirmo and Record, 1990). To investigate the temperature dependency of \( P_{spoIIG} \), transcription assays were carried out at temperatures ranging from 12° to 37° C. Figure 12 shows that transcription from linearized \( P_{spoIIG} \) template was extremely sensitive to temperature change, even in the
Figure 11. Effect of intermediate levels of Spo0A phosphorylation on transcription from

\( P_{spoIIG} \).

Samples removed from a phosphorelay reaction at various times after kinase activity
had been initiated, were added to \( P_{spoIIG} \) transcription assays.

(A) The increase in Spo0A phosphorylation over time, was followed by sampling a
phosphorelay reaction that contained \([\gamma^{32}P]ATP\). The samples were immediately
mixed with protein gel loading buffer to terminate kinase activity and were later
electrophoresed through a 15% SDS - polyacrylamide gel (Materials and Methods V, 1).
The \( 32P \) - labeled protein bands were localized by autoradiography.

(B) Time-course samples taken from a non-radioactive phosphorelay reaction were
immediately added to \( P_{spoIIG} \) transcription assays (Materials and Methods V, 2). The
\( 32P \) - labeled transcripts produced in these assays were separated by PAGE and detected
by autoradiography.

(C) Bands containing \( 32P \) - labeled Spo0A protein or \( P_{spoIIG} \) transcripts were excised
from polyacrylamide gels and level of Cerenkov radiation in the gel slices was measured
to allow quantitation of Spo0A phosphorylation (-○-) or \( P_{spoIIG} \) activity (-●-)
(Materials and Methods IV, 5). These values are plotted as a function of time.
Figure 12. Temperature sensitivity of transcription from $P_{spolIG}$.

$P_{spolIG}$ transcription assays were carried out at various temperatures. Transcription was quantitated by measuring Cerenkov radiation in gel slices containing $P_{spolIG}$ transcripts which had been separated by electrophoresis through a 6% urea-polyacrylamide gel and detected by autoradiography (Materials and Methods IV, 5). Promoter activity measured in transcription assays that contained no Spo0A (Δ), or 200 nM Spo0A (○) or Spo0A-P (●), was plotted as a function of temperature.
presence of Spo0A-P. \( P_{\text{spoII}G} \) activity in transcription assays containing 200 nM Spo0A or Spo0A-P, declined sharply at lower temperatures and was essentially zero below 27° C.

2. The effect of ion concentration on \( P_{\text{spoII}G} \) transcription.

In vitro transcription is normally very sensitive to salt concentration (Shaner et al., 1983; Roe et al., 1984; Leirmo and Record, 1990). This salt sensitivity results because of the high negative charge density of DNA molecules. In an aqueous salt solution, cations accumulate next to DNA molecules forming a steep ion concentration gradient when compared to the electrolyte concentration in bulk solution. This phenomenon has been termed the 'polyelectrolyte effect' (Lohman, 1985; Leirmo and Record, 1990). The accumulation of cations (or counterions), is essentially independent of total salt concentration and in the presence of monovalent cations, 88% of the charge associated with the phosphate groups of the DNA is effectively neutralized (Lohman, 1985). When RNA polymerase binds to promoter DNA, or melts the helix, counterions are displaced into the bulk solution providing a substantial entropic contribution to the transcription initiation reaction (Lohman et al., 1978; Shaner et al., 1983; Lohman, 1985).

Transcription is favored under conditions of low salt concentration because the steeper ion gradient provides a larger increase in entropy.

To examine the salt dependency of \( P_{\text{spoII}G} \), increasing concentrations of NaCl were added to transcription assays. When the salt was added prior to RNA polymerase \( P_{\text{spoII}G} \) transcription was abolished at NaCl concentrations of 100 mM and above (Figure 13A). This effect was observed whether Spo0A-P was added to transcription assays or not. When NaCl was added to assays after RNA polymerase and initiating nucleotides had been incubated with \( P_{\text{spoII}G} \), no salt sensitivity was observed at any NaCl concentration (Figure 13B). It was concluded that increased NaCl concentration adversely affected the process of transcription initiation at \( P_{\text{spoII}G} \) and that the stimulatory effect of Spo0A-P could not compensate
Figure 13. Influence of NaCl concentration on transcription from \( P_{\text{spoIIG}} \).

To determine whether NaCl affected the initiation of RNA synthesis, the salt was added to mixtures of DNA, ATP, GTP and buffer before beginning an initiation reaction with the addition of RNA polymerase (A), or by adding the salt simultaneously with initiating nucleotides after polymerase had been incubated with promoter template (B). Completed transcription reactions were electrophoresed through 6% polyacrylamide gels and \(^{32}\)P-labeled transcripts were detected by autoradiography. NaCl concentrations added to each reaction were; lane 0, no NaCl added; lane 1, 50mM; lane 2, 100 mM; lane 3, 200 mM; lane 4, 300 mM; lane 5, 400 mM; lane 6, 500 mM. Reactions which contained either 200 nM SpoOA or SpoOA-P are indicated.
for the inhibition. In addition, it appeared that RNA polymerase in its heparin-resistant state was insensitive to salt inhibition. Similar salt effects were observed when KCl was substituted for NaCl (Figure 14). Although it appeared that transcription initiation assays that contained Spo0A-P tolerated small increases in KCl concentration, transcription still diminished rapidly as the KCl concentration approached 100 mM. In contrast, assays that did not contain Spo0A-P exhibited little or no transcription at 20 mM KCl and above.

It has been reported that the salt sensitivity of in vitro transcription can be modulated by the replacement of Cl− with organic anions such as glutamate or acetate (Leirimo et al., 1987; Ha et al., 1992). Transcription assays containing various inputs of potassium acetate (KAc) were carried out to examine its effect on $P_{spoIIG}$ activity (Figure 15). This experiment demonstrated dramatic differences in the salt sensitivity of transcription assays that contained either no Spo0A, Spo0A or Spo0A-P. Transcription in assays that contained no Spo0A, or non-phosphorylated Spo0A, decreased sharply with increased concentrations of KAc. However, promoter activity in assays that contained Spo0A-P was only slightly affected. Although the molecular basis for the anion effect on transcription initiation is not known (Ha et al., 1992), it was concluded that changes in KAc concentration might be used to probe differences in $P_{spoIIG}$ transcription stimulation by the various forms of the Spo0A protein (Results IV, 10).

IV. Investigation into the mechanism of transcription activation by Spo0A-P.

1. $P_{spoIIG}$ transcription rate assays.

Once it had been established that Spo0A-P enhanced $P_{spoIIG}$ transcriptional activity in vitro, studies turned to an examination of the stimulation mechanism. The purpose of these investigations was to determine whether $P_{spoIIG}$ stimulation was accompanied by an increase in the rate of transcription initiation. Transcription rate assays were conducted by adding RNA polymerase to a mixture of $P_{spoIIG}$ template, ATP and GTP
Figure 14. Effect of KCl concentration on $P_{spoIIG}$ transcription.

KCl was added to transcription assays prior to RNA polymerase. Final salt concentrations in each assay was; lane 0, no KCl added; lane 1, 20 mM KCl; lane 2, 40 mM KCl; lane 3, 60 mM KCl; lane 4, 80 mM KCl; lane 5, 100 mM KCl. Reactions carried out in the absence of Spo0A, or with 200 nM Spo0A-P are indicated.
Figure 15. Effect of increasing KAc concentration on \( P_{\text{spoII}G} \) activity.

Autoradiogram of a polyacrylamide gel showing \( P_{\text{spoII}G} \) transcripts generated in transcription assays that contained various KAc concentrations without Spo0A, or in the presence of 400 nM Spo0A(-P). KAc was added to individual transcription assays prior to RNA polymerase. Final salt concentrations in each assays was; lane 0, no KAc added; lane 1, 20mM; lane 2, 40 mM; lane 3, 60 mM; lane 4, 80 mM; lane 5, 100 mM; lane 6, 120 mM. Reactions that contained no Spo0A, Spo0A or Spo0A-P are indicated.
to begin a transcription initiation reaction. At regular intervals, samples were removed and added to a mixture of heparin, CTP, and UTP to block the initiation reaction but allow RNA elongation from heparin-resistant complexes. The production of full length transcripts in each sample was assumed to be proportional to the number of heparin-resistant complexes formed at the time the assay was sampled. Therefore, these assays measured the rate of heparin-resistant complex formation. The correlation between heparin-resistance and the initiation of RNA synthesis (Results I, 2), indicated that rates of complex formation were equivalent to rates of transcription initiation.

Figure 16 shows a time-course of heparin-resistant complex formation in rate assays that contained no Spo0A, or 400 nM Spo0A-P. Two conclusions were derived from this plot. First, although the maximum levels of transcription were very different in each assay, heparin-resistant complex formation appeared to be complete within 10 - 15 minutes of adding RNA polymerase. Secondly, the rate of approach to maximum transcription appeared to be significantly faster in the assay that contained Spo0A-P.

2. Measurement of rate constants for $P_{spolII}$ transcription initiation.

Because RNA polymerase required initiating nucleotides to reach the heparin-resistant state, it was apparent that at least one heparin-sensitive intermediate preceded its formation. Since the assay used to determine the rate of initiation at $P_{spolII}$ detected only heparin-resistant complexes, the production of these complexes may be described as a single step isomerization;

$$\text{HS} \xrightleftharpoons[k_f]{k_r} \text{HR} \quad (\text{R1})$$

where HS is a heparin-sensitive intermediate that converts to HR, a heparin-resistant complex. The observed rate of HR formation ($k_{obs}$) is a function of the association ($k_f$) and dissociation ($k_r$) rate constants and will follow first-order kinetics if the overall
A time-course of heparin-resistant complex formation was begun by adding RNA polymerase to mixtures of $P_{\text{spolIG}}$ template, initiating NTPs (ATP and GTP), and either no Spo0A (○) or 400 nM Spo0A-P (●) (Materials and Methods V, 1). At various times samples were removed and added to a solution of heparin, CTP, and UTP to stop the reaction while allowing transcript elongation from heparin-resistant complexes. The $^{32}$P-labeled RNA was separated by electrophoresis through a 6% urea - polyacrylamide gel and promoter activity was quantitated by measuring Cerenkov radiation in transcript bands localized by autoradiography (Materials and Methods IV, 5).
reaction is dominated by a single slow step, and if all the reaction steps are either true or pseudo first-order.

When transcription rate assays are carried out under conditions of excess RNA polymerase concentration, HR formation should follow pseudo first-order kinetics. Thus, the isomerization of HS complexes may be treated as a first-order decay reaction with the rate of approach to maximum HR formation given by the equation;

\[
\frac{d[HR]}{dt} = -(k_f + k_r)[HP] + k_f[P_T]
\]  \hspace{1cm} (R2)

where \([P_T]\) is the total concentration of heparin-sensitive and heparin-resistant complexes (Appendix I, 3). A general solution to equation (3) is given by the expression;

\[
\ln\left(1 - \frac{[HR]}{[HR]_\infty}\right) = -\left(\frac{1}{\tau}\right)t
\]  \hspace{1cm} (R3)

where \(\tau\) (tau) is equivalent to \(1/k_{obs}\), and \([HR]_\infty\) is the final HR concentration at reaction completion (Appendix I, 3). Therefore, a plot of \(\ln(1 - [HR]/[HR]_\infty)\) versus time \((k_{obs}\) plot) should yield a straight line with a slope equal to the negative of the observed rate constant \((- (1/\tau))\), for the overall process of heparin-resistant complex formation.

Two additional aspects of \(k_{obs}\) plots should be considered. Rate constants derived from \(k_{obs}\) plots are independent of the completion levels of HR formation in different assays. Therefore, \(k_{obs}\) values obtained from assays that contained no Spo0A or Spo0A-P could be directly compared to assess how the rate of HR formation was affected. Lastly, if the overall production of HR is dominated by a single slow, or rate limiting step, then \(k_{obs}\) plots should produce a straight line regardless of the actual number of reaction steps involved. Consequently, \(\tau\) measurements did not require a detailed understanding of the actual reaction mechanism for HR production.
3. Tau analysis.

Rates of transcription initiation for many *E. coli* promoters have been investigated by tau analysis (McClure, 1980; Hawley and McClure, 1980; Stefano and Gralla, 1982; Giladi et al., 1992). The analysis has allowed investigators to probe individual steps of the initiation process by observing how changes to various parameters (including temperature, ion concentration, or promoter mutations) affect specific rate constants (Stefano and Gralla, 1982; Roe et al., 1984; Roe et al., 1985). A tau analysis of *P*<sub>spoIIG</sub> transcription initiation was undertaken to identify which reaction step was affected by Spo0A-P.

The process of heparin-resistant complex formation at *P*<sub>spoIIG</sub> appeared to involve at least two reaction steps. These are represented by the model:

$$ R + P \stackrel{k_1}{\rightarrow} HS \stackrel{k_r}{\rightarrow} HR $$

where RNA polymerase and *P*<sub>spoIIG</sub> combine to form a heparin-sensitive complex followed by an isomerization to the heparin-resistant state. Under conditions of excess RNA polymerase concentration the model is reduced to

$$ P \stackrel{[R]k_1}{\rightarrow} HS \stackrel{k_r}{\rightarrow} HR $$

(Appendix I, 4). By applying the steady state assumption (that the change in [HS] is negligible) it can be shown that the rate of HR formation is described by expression (5);

$$ \frac{d[HR]}{dt} = k_r [HS] - k_r [HR] $$

(R6)
which can be rearranged to;

\[
\frac{d[HR]}{dt} = -\left(\frac{1}{\tau}\right)[HR] + \beta \quad (R7)
\]

(Appendix I, 3).

A solution for \(1/\tau\) depends on the reaction mechanism (Strickland et al., 1975).

According to the two step reaction in model (R4);

\[
1/\tau = \frac{k_1[R](k_f + k_r) + k_1 k_r}{k_1[R] + k_{-1} + k_f} \quad (R8)
\]

Given the assumption that \(k_f\) is negligible compared to \(k_f\) which is indicated by the stability of heparin-resistant complexes, this expression can be reduced to;

\[
1/\tau = \frac{k_1[R] k_f}{k_1[R] + k_{-1} + k_f} \quad (R9)
\]

or the reciprocal form;

\[
\tau = \frac{1}{k_{obs}} = \frac{k_{-1} + k_f}{k_f k_1} \left(\frac{1}{[R]}\right) + \frac{1}{k_f} \quad (R10)
\]

Thus a plot of tau versus \(1/[R]\) should yield a straight line with an ordinate intercept equal to \(1/k_f\) and a slope of \((k_{-1} + k_f) / k_f k_1\).
Figure 17. Effect of Spo0A concentration on $P_{\text{spolIG}}$ transcription rate assays.

Transcription rate assays were begun by adding polymerase to mixtures of $P_{\text{spolIG}}$ template, ATP, GTP and either no Spo0A (A) or 400 nM Spo0A-P (B). At regular intervals samples were removed and added to a mixture of heparin, CTP and UTP to block the reaction and facilitate elongation of full length transcripts from heparin-resistant complexes. Two 15 minute time-point samples were taken from each initiation reaction to measure completion levels of transcription. The $^{32}$P-labeled RNA in each sample was separated by PAGE and detected by autoradiography (shown above). Promoter activity in time-point and completion samples were quantitated (Materials and Methods IV, 5) and used to calculate overall rate constants for each transcription initiation reaction (see Figure 18).
Figure 18. Example k_{obs} plots from P_{spolig} rate assays that contained various RNA polymerase concentrations.

\(^{32}\text{P}\) - labeled RNA from transcription rate assays was separated by PAGE and localized by autoradiography. RNA levels were quantitated by measuring Cerenkov radiation in transcript bands separated by polyacrylamide gel electrophoresis (Figure 17; Methods and Materials IV, 5). These levels were assumed to be proportional to the concentration of heparin-resistant complexes present in time-point ([HR]) or completion ([HR]_\infty) samples. The rate of approach to rate assay completion was determined by expressing the level of transcription in time-point samples as \([HR]/[HR]_\infty\) (HR*), and the data was plotted as ln(1 - HR*) versus time (HR* = [HR]/[HR]_\infty) (Results IV, 2). Rate constants for the overall process of heparin-resistant complex formation (k_{obs}) were calculated from the slopes of lines drawn through data sets using linear least-squares analysis. In general, the coefficient constant for these lines was 0.8 or greater. The k_{obs} plots shown here are from rate assays that contained polymerase concentrations of 11 nM (▲), 14 nM (△), 20 nM (●) and 33 nM (○). These assays contained either no Spo0A (A) or 400 nM Spo0A-P (B).
Table I. $k_{\text{obs}}$ values measured for $P_{\text{spoIIG}}$ transcription rate assays that contained different RNA polymerase concentrations.

The $k_{\text{obs}}$ and tau ($1/k_{\text{obs}}$) values shown below summarize results from separate experiments. In some cases, measures of $k_{\text{obs}}$ and tau were averaged from multiple assays conducted at a given RNA polymerase concentration. These values are plotted in Figure 19.

<table>
<thead>
<tr>
<th>RNA polymerase concentration (μM)</th>
<th>averaged $k_{\text{obs}}$ (sec$^{-1} \times 10^2$)</th>
<th>tau (sec)</th>
</tr>
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<tr>
<td>no Spo0A</td>
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<td></td>
</tr>
<tr>
<td>0.008</td>
<td>0.376 (1)</td>
<td>266 ± 80</td>
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<tr>
<td>0.010</td>
<td>0.313 (3)</td>
<td>319 ± 120</td>
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<td>0.011</td>
<td>0.340 (1)</td>
<td>294 ± 88</td>
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<td>0.013</td>
<td>0.235 (2)</td>
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<tr>
<td>0.014</td>
<td>0.326 (1)</td>
<td>306 ± 92</td>
</tr>
<tr>
<td>0.017</td>
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<td>284 ± 131</td>
</tr>
<tr>
<td>0.020</td>
<td>0.353 (2)</td>
<td>283 ± 56</td>
</tr>
<tr>
<td>0.025</td>
<td>0.348 (2)</td>
<td>287 ± 221</td>
</tr>
<tr>
<td>400 nM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spo0A-P</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>1.58 (1)</td>
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</tr>
<tr>
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<td>80.0 ± 30</td>
</tr>
<tr>
<td>0.025</td>
<td>1.17 (1)</td>
<td>85.5 ± 26</td>
</tr>
</tbody>
</table>

Note: figures in parentheses denote number of times experiment was repeated at the given RNA polymerase concentration.
Figure 19. *P._spoilG* tau plot.

The tau values shown in Table I are plotted as a function of the reciprocal RNA polymerase concentration. Lines for data sets obtained from rate assays that contained no Spo0A (○), or 400 nM Spo0A-P (●), were calculated by linear least-square analysis.
4. The $P_{spolIG}$ tau plot.

The mechanism of transcription initiation at $P_{spolIG}$ was investigated by measuring $k_{obs}$ for rate assays that contained various RNA polymerase concentrations that were at least 5 fold greater than the concentration of $P_{spolIG}$ template. These assays were conducted with and without phosphorelay samples to observe the effect of Spo0A-P on the initiation reaction (Figure 17). Rate constants were determined from $k_{obs}$ plots like those shown in Figure 18. Because these plots suggested straight lines, it was concluded that the RNA polymerase concentrations used in these experiments were sufficiently high to allow heparin-resistant complex formation to follow pseudo first-order kinetics. In addition, the steeper slopes of the lines representing $k_{obs}$ values for transcription assays that contained Spo0A-P, indicated that the factor stimulated a rate limiting step in the production of heparin-resistant complexes at $P_{spolIG}$.

$k_{obs}$ values measured for several transcription assays conducted with and without phosphorelay aliquots, were averaged (Table I) and plotted as tau versus the reciprocal of the RNA polymerase concentration (Figure 19). The slopes of the lines calculated for this plot, appeared to be zero suggesting that the rate of transcription initiation at $P_{spolIG}$ was independent of RNA polymerase concentration in the presence or absence of Spo0A-P. The obvious effect of Spo0A-P was to cause a substantial downward shift of the ordinate intercept suggesting a significant increase to $k_f$. Thus, Spo0A-P had apparently influenced the rate $P_{spolIG}$ transcription by catalyzing an isomerization step rather than affecting the binding of polymerase to the promoter.

In considering equation (R10) (Results IV, 3), a lack of rate dependence on RNA polymerase concentration indicated that the ratio $(k_{-1} + k_f)/k_1 k_f$ was very small. If it is assumed that $k_{-1}$ is much greater than $k_f$ the slope of a tau plot becomes $1/K_B k_f$, where $K_B$ is the equilibrium binding constant for the bimolecular association of the polymerase and promoter ($K_B = k_1/k_{-1}$). A slope $1/K_B k_f$ would be expected for a transcription initiation process referred to as a rapid equilibrium mechanism (Roe et al., 1984; Roe et
According to this mechanism, if $k_1/k_{-1} >> k_f$, binding of RNA polymerase to $P_{spoIIG}$ would equilibrate very rapidly relative to a forward isomerization step. Thus, the overall rate of transcription initiation would be dominated by the unimolecular isomerization of HS to HR, and $\tau$ would be independent of polymerase concentration.

5. Effect of DNA concentration on rate of initiation at $P_{spoIIG}$.

Rate studies of *E. coli* promoters have revealed that $\tau$ values are not affected by changes in promoter template concentration (Stefano and Gralla, 1980). It has been proposed that there is no rate dependence on template concentration because virtually all the RNA polymerase added to transcription assays is quickly bound to promoter and non-specific sites on the DNA. Stefano and Gralla (1980) proposed that rates of polymerase transfer between different sites is not affected by increased template concentration because the ratio of promoters to non-specific sites is not altered. To examine whether rates of heparin-resistant complex formation at $P_{spoIIG}$ were also unaffected by promoter concentration, transcription rate assays were carried out at various $P_{spoIIG}$ template concentrations. The $k_{\text{obs}}$ values obtained from these experiments are plotted as a function of $P_{spoIIG}$ concentration in Figure 20. The plot demonstrates that $k_{\text{obs}}$ values did not change with increased concentrations of DNA, therefore, the rate of transcription initiation at $P_{spoIIG}$ was independent of promoter concentration.

6. Effect of GTP concentration on initiation rates at $P_{spoIIG}$.

Normally, a final concentration of 10 $\mu$M GTP was added to $P_{spoIIG}$ transcription assays when [$\gamma^{32}\text{P}$]-GTP was used to label RNA transcripts (Materials and Methods IV, 3). However, it became a concern that low GTP concentrations could influence rate assays, particularly when they contained Spo0A-P where high levels of transcription initiation might reduce GTP concentration to the point where heparin-resistant complex formation was affected. To test this possibility, $k_{\text{obs}}$ values were measured for two rate assays containing 400 nM Spo0A-P and either 10 or 40 $\mu$M GTP (Figure 21). Since the
overall rate of initiation in the two assays was essentially identical, it was concluded that 10 μM GTP was an adequate concentration for conducting transcription assays without affecting rates of initiation.

7. Effect of Spo0A(-P) concentration on the rate of initiation at $P_{spoII}G$.

Since 400 nM Spo0A-P had been observed to stimulate the rate of transcription initiation at $P_{spoII}G$ (Figures 16 & 19), various dilutions of phosphorelay samples were tested in rate assays to examine the relationship between the rate of heparin-resistant complex formation and Spo0A(-P) concentration. A plot of completion levels of $P_{spoII}G$ activity versus Spo0A(-P) concentration is shown in Figure 22 and demonstrates that stimulation of $P_{spoII}G$ transcription reached a plateau at 200 nM Spo0A-P. Increased concentrations of non-phosphorylated Spo0A affected maximum levels of transcription only slightly.

As evidenced by the increasingly steeper slopes of the $k_{obs}$ plot in Figure 23B, the rate of initiation at $P_{spoII}G$ increased dramatically as Spo0A-P concentration was raised. A plot of $k_{obs}$ versus Spo0A(-P) concentration (Figure 24A) demonstrates that unlike the effect on completion levels of transcription, stimulation of initiation rates did not plateau over the range of Spo0A-P concentrations tested. Instead rates of transcription initiation at $P_{spoII}G$ continued to accelerate. Increased concentrations of non-phosphorylated Spo0A also stimulated the rate of initiation at $P_{spoII}G$, however, its influence was minimal compared to Spo0A-P. When the data from Figure 24A was converted to a double reciprocal plot (Figure 24B) it demonstrated a linear relationship between the stimulation to the rate of transcription initiation at $P_{spoII}G$ and the concentration of Spo0A(-P).

8. Interpretation of the effect of Spo0A-P on the rate of initiation at $P_{spoII}G$.

Since all the lines in the $k_{obs}$ plot of Figure 23B are straight, it appeared Spo0A-P had stimulated the rate of transcription initiation at $P_{spoII}G$ by affecting a rate limiting reaction step. The downward shift in the ordinate intercept of the tau plot (Figure 19)
Figure 20. Effect of $P_{spoIIG}$ template concentration on rates of transcription initiation.

(A) To examine whether promoter concentration affected the rate of transcription initiation, rate assays were carried out at four $P_{spoIIG}$ concentrations. The assays contained no Spo0A (open symbols) or 400 nM Spo0A-P (closed symbols). DNA concentrations were 2 nM ($\triangle$); 4 nM ($\square$); 6 nM ($\circ$); or 8 nM ($\ast$). The slopes of lines through each data set were calculated by linear least squares analysis. In general, the coefficient constant for these lines was 0.8 or greater. The rate values derived from these slopes are shown in Table II.

(B) Rate constants derived from the $k_{obs}$ plot shown in Figure 20A (shown above) are plotted as a function of $P_{spoIIG}$ template concentration. The data is from rate assays that contained no Spo0A ($\bigcirc$), or 400 nM Spo0A-P ($\bullet$).
Table II. $k_{\text{obs}}$ values obtained from transcription assays that contained various $P_{\text{spoIIG}}$ template concentrations.

Transcription rate assays were carried out at different $P_{\text{spoIIG}}$ template concentrations to determine whether the rate of heparin-resistant complex formation was affected. Assays were carried out in the absence or presence of 400 nM Spo0A-P. The rate values shown below were derived from the $k_{\text{obs}}$ plot shown in panel A of Figure 20.

<table>
<thead>
<tr>
<th>$[P_{\text{spoIIG}}]$ (nM)</th>
<th>+/- Spo0A-P</th>
<th>$k_{\text{obs}}$ (sec$^{-1} \times 10^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 (▲)</td>
<td>-</td>
<td>0.181</td>
</tr>
<tr>
<td>4 (▲)</td>
<td>-</td>
<td>0.192</td>
</tr>
<tr>
<td>6 (●)</td>
<td>-</td>
<td>0.229</td>
</tr>
<tr>
<td>8 (●)</td>
<td>-</td>
<td>0.160</td>
</tr>
<tr>
<td>2 (▲)</td>
<td>+</td>
<td>2.01</td>
</tr>
<tr>
<td>4 (■)</td>
<td>+</td>
<td>1.71</td>
</tr>
<tr>
<td>6 (●)</td>
<td>+</td>
<td>1.74</td>
</tr>
<tr>
<td>8 (●)</td>
<td>+</td>
<td>1.76</td>
</tr>
</tbody>
</table>
Figure 21. Effect of GTP concentration on the rate of $P_{spoHG}$ transcription initiation.

To determine whether the low GTP concentration used in $P_{spoHG}$ transcription assays affected the rate of heparin-resistant complex formation, two assays were carried out at GTP concentrations that differed by four fold. Both reactions contained 400 nM Spo0A-P. The overall rate constants derived from the $k_{obs}$ plot shown here were $2.28 \times 10^2$ and $2.00 \times 10^2$ sec$^{-1}$, for assays that contained 10 and 40 $\mu$M GTP, respectively.
Completion levels of $P_{spoIIG}$ promoter activity in rate assays that contained increasing inputs of Spo0A (-○-) or Spo0A-P (-●-), are plotted as a function of total Spo0A protein concentration. Completion levels were determined by averaging promoter activity measured in two samples taken from rate assays 15 minutes after initiating heparin-complex formation with the addition of RNA polymerase (Figure 17).
Figure 23. Determination of rate constants for assays containing various Spo0A(-P) concentrations.

A series of rate assays that contained increasing concentrations of either Spo0A (A) or Spo0A-P (B), were carried out to determine whether rates of $P_{spoIIG}$ transcription initiation would be affected. Rate constants for heparin-resistant complex formation were derived from the slopes of straight lines calculated for the data sets in the $k_{obs}$ plots shown here. These values and their reciprocals ($\tau$), are given in Table III.
Table III. Rate constants for heparin-resistant complex formation in $P_{spoIIIC}$ transcription assays that contained various concentrations of Spo0A(-P).

Rate constants derived from the $k_{obs}$ plots shown in Figure 23 are shown below along with their corresponding tau values. The tau values are plotted in Figure 24, panel B.

<table>
<thead>
<tr>
<th>[Spo0A(-P)] (nM)</th>
<th>$k_{obs}$ (sec$^{-1}$ x 10$^2$)</th>
<th>tau (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (•)</td>
<td>0.409</td>
<td>244 ± 73</td>
</tr>
<tr>
<td>50 (Δ)</td>
<td>0.413</td>
<td>242 ± 73</td>
</tr>
<tr>
<td>100 (□)</td>
<td>0.430</td>
<td>233 ± 70</td>
</tr>
<tr>
<td>200 (◊)</td>
<td>0.567</td>
<td>176 ± 53</td>
</tr>
<tr>
<td>400 (●)</td>
<td>0.861</td>
<td>116 ± 35</td>
</tr>
<tr>
<td>800 (■)</td>
<td>0.601</td>
<td>166 ± 50</td>
</tr>
</tbody>
</table>

| **B**            |                                 |           |
| 0 (•)            | 0.409                           | 244 ± 73  |
| 50 (Δ)           | 0.811                           | 123 ± 37  |
| 100 (□)          | 1.60                            | 62.5 ± 19 |
| 200 (◊)          | 2.68                            | 37.3 ± 11 |
| 400 (●)          | 3.02                            | 33.1 ± 10 |
| 800 (■)          | 3.81                            | 26.2 ± 8  |
for reactions that contained Spo0A-P, indicated that Spo0A-P had catalyzed a step that occurred after binding of polymerase to the promoter. This effect was examined by considering the reaction model;

\[
\begin{align*}
R + P & \xrightleftharpoons[k_1]{k_{-1}} HS_1 + OA(-P) \xrightleftharpoons[k_2]{k_{-2}} HS_2 \xrightarrow[k_f]{k_{r}} HR \\
\end{align*}
\]  

(R11)

where HS_1 and HS_2 are different heparin-sensitive intermediates resulting from RNA polymerase binding to \( P_{spoIIG} \) (HS_1) and interacting with Spo0A(-P) (HS_2). The order of polymerase or Spo0A(-P) binding to the DNA is not specified in this model so that polymerase could bind \( P_{spoIIG} \) and then interact with Spo0A(-P) bound to specific OA boxes, or visa versa. The model also does not preclude the possibility that there may be additional heparin-sensitive complexes formed during the isomerization to a heparin-resistant state. It appeared that binding of RNA polymerase to \( P_{spoIIG} \) equilibrated so rapidly that this step did not influence the overall rate of transcription initiation, therefore, a rate dependence on Spo0A(-P) apparently results because a slow isomerization from HS_2 to HR is affected.

The effect of Spo0A-P concentration on rate of initiation was evaluated using a form of tau analysis derived from equation (8), where HS_1 and Spo0A(-P) were treated as the binding site and ligand, respectively, to give the expression;

\[
\tau = \frac{k_2 + k_f}{k_2 k_f} \left( \frac{1}{[OA(-P)]} \right) + \frac{1}{k_f} . \quad \text{(R12)}
\]

In this case, a plot of tau versus the reciprocal of the Spo0A(-P) concentration would yield an ordinate intercept equal to the inverse of the rate constant for isomerization of HS_2. The \( k_f \) values determined from Figure 24B were 0.061 sec\(^{-1}\) and 0.007 sec\(^{-1}\) for
Figure 24. Effect of SpoOA(-P) concentration on rates of \( P_{spoHG} \) transcription initiation.

(A) The \( k_{\text{obs}} \) values from rate assays that contained various concentrations of SpoOA(-P) (Figure 23, Table III), are plotted as a function of SpoOA (-○-) or SpoOA-P (■-) concentration.

(B) Tau values (1/k\text{obs}) from transcription rate assays in part A are plotted as a function of the reciprocal of the total SpoOA concentration. Lines through each data set were calculated by linear least-squares analysis. Ordinate intercepts, corresponding to the forward isomerization rate constant, \( k_f \), (Results IV, 3) were 0.701 x 10\(^2\) sec\(^{-1}\) for Spo0A (-○-) and 6.10 x 10\(^2\) sec\(^{-1}\) for Spo0A-P (■-).
reactions that contained Spo0A-P or Spo0A, respectively. Thus, phosphorylation of Spo0A increased stimulation of the rate of heparin-resistant complex formation nearly 10 fold.

9. The effect of the Spo0A binding domain polypeptide (Spo0A^{BD}) on \( P_{spoIIG} \) transcription.

A trypsin digest of the Spo0A protein produces two polypeptides approximately 13 and 17 kDa in size (Grimsley et al., 1994). The larger fragment (Spo0A^{BD}) comprises the C-terminal portion of the protein known to include the DNA binding domain, but does not contain the aspartate^{56} residue which is the phospho-acceptor site (Burbulis et al., 1991). To determine whether Spo0A^{BD} could influence \( P_{spoIIG} \) activity in vitro, it was added to transcription rate assays using the same procedure as for non-phosphorylated Spo0A. Figure 25A shows the completion levels of transcription obtained from rate assays conducted at various Spo0A^{BD} concentrations. The plot demonstrates a linear relationship between maximum promoter activity and Spo0A^{BD} concentration. The magnitude of stimulation by Spo0A^{BD} appeared to be less than the stimulation mediated by similar concentrations of Spo0A-P. However, a direct comparison of the effects of Spo0A-P and Spo0A^{BD} is difficult because of the inability to determine the specific activity of the proteins. Based on similar concentrations of total protein, it appeared that the effect of Spo0A^{BD} on \( P_{spoIIG} \) transcription was intermediate to those of intact non-phosphorylated Spo0A and Spo0A-P.

Values for the rate of approach to maximum transcription in rate assays that contained Spo0A^{BD} were calculated and plotted as a function of polypeptide concentration (Figure 25B). The increase to \( k_{obs} \) in reactions that contained 50 nM or more Spo0A^{BD}, appeared to be directly proportional to Spo0A^{BD} concentration. A plot of \( \tau \) versus \( 1/[spo0A^{BD}] \) (Figure 26) indicated that Spo0A^{BD} concentrations below 50 nM were too low to cause a measurable effect on \( k_{obs} \). However, interpolation of the slope calculated for rates measured at 100 nM Spo0A^{BD} and above, corresponded to an
ordinant intercept that indicated a $k_f$ value of 0.027 sec$^{-1}$. This estimate was almost half the value determined from experiments with Spo0A-P and implied that removal of the N-terminal portion of the Spo0A protein may have activated its ability to stimulate $P_{spoII}G$ transcription. This raises the possibility that one function of the N-terminal domain is to inhibit the transcription activating property of the DNA binding domain. The role of Spo0A phosphorylation could be to neutralize this inhibition.

10. The KAc effect on rates of initiation at $P_{spoII}G$.

Since increased KAc concentration was observed to have an inhibitory effect on $P_{spoII}G$ activity except in the presence of Spo0A-P, the effect of KAc concentration on the rate of initiation at $P_{spoII}G$ was examined. Transcription assays containing no Spo0A, or 400 nM Spo0A, Spo0A$^{BD}$ or Spo0A-P, were carried out at KAc concentrations that ranged from 0 to 120 mM. Completion levels of transcription from this experiment are plotted as a function of [KAc] in Figure 27. This plot shows that increased KAc concentrations lowered maximum transcription in all assays except those containing Spo0A-P. The most dramatic inhibition of transcription occurred in assays containing Spo0A$^{BD}$. In this case, promoter activity in the absence of KAc was similar to transcription levels obtained with Spo0A-P. However, raising KAc concentration to 80 or 120 mM in assays that contained Spo0A$^{BD}$ caused a seven fold decline in transcription to a level that was similar to assays containing no Spo0A. Maximum promoter activity was also inhibited by elevated KAc concentration in rate assays that contained no Spo0A or non-phosphorylated protein but the effect was much less pronounced.

Rate constants from transcription assays that contained various forms of Spo0A at different KAc concentrations, are summarized in Table IV. While the rates measured in assays that had no Spo0A or Spo0A-P were largely unaffected by elevated KAc concentrations, it is apparent that the ability of either Spo0A or Spo0A$^{BD}$ to stimulate the rate of initiation at $P_{spoII}G$, was markedly diminished under this condition.
Figure 25. Effect of Spo0A\textsuperscript{BD} on completion levels and rate of $P_{spoIIG}$ transcription initiation.

To examine the ability of Spo0A\textsuperscript{BD} to stimulate $P_{spoIIG}$ transcription, various concentrations of the polypeptide were added to transcription rate assays as described for experiments involving the intact protein (Materials and Methods IV, 4). The effect of Spo0A\textsuperscript{BD} was examined by plotting completion levels (A), or rate constants ($k_{obs}$ values) (B), as a function of total Spo0A\textsuperscript{BD} protein concentration.
Figure 26. Tau analysis of the effect of Spo0A^{BD}.

The inverse of $k_{obs}$ values (tau) shown in Figure 25B are plotted as a function of reciprocal Spo0A^{BD} concentration. The line drawn was considered to be the best fit to the data.
The effect of KAc on the rate of $P_{spoIG}$ transcription initiation, was tested by adding various concentrations of the salt to rate assays that contained no Spo0A (−□−), or 400 nM Spo0A (−○−), Spo0A<sup>BD</sup> (−Δ−) or Spo0A-P (−●−). Various concentrations of KAc were mixed with $P_{spoIG}$ template, ATP and GTP, before beginning an initiation time-course reaction with the addition of RNA polymerase (Materials and Methods IV, 4). The completion levels of transcription represented by the averaged level of transcription in two 15 minute time samples, are plotted as a function of KAc concentration.
Differences in the abilities of the various forms of Spo0A to stimulate \( P_{\text{spolIG}} \) transcription was further illustrated when initial rates of heparin-resistant complex formation were examined. A plot of the number of \( P_{\text{spolIG}} \) transcripts measured in rate assays that contained 120 mM KAc reveals the combined effects to both the completion level and rate of complex formation (Figure 28). In the absence of KAc, Spo0A-P stimulated an 8 - 10 fold increase in the rate of heparin-complex formation compared to assays that contained no Spo0A or non-phosphorylated protein (Figure 28A). The reaction that contained Spo0A\(^{\text{BD}}\) exhibited a pronounced lag prior to reaching a constant rate of complex formation that was approximately half that of Spo0A-P. The reason for this lag is not known, however, it could reflect differences in the binding affinities of Spo0A\(^{\text{BD}}\) and intact protein, or suggest that the polypeptide lacked a portion of the activating domain required for optimum transcription stimulation. In either instance, the lag suggested that two reaction steps contributed significantly to the overall transcription initiation in rate assays that contained Spo0A\(^{\text{BD}}\).

A plot of transcripts versus time for rate assays containing 120 mM KAc clearly demonstrates that the ability of Spo0A-P to stimulate \( P_{\text{spolIG}} \) was far superior compared to other forms of the protein (Table V, Figure 28B). The initial rate of heparin-resistant complex formation in the presence of Spo0A-P was approximately 35 times greater than in assays containing Spo0A or Spo0A\(^{\text{BD}}\), and was more than 70 times the rate observed without Spo0A protein.

11. Rate of transcription initiation from a mutant form of \( P_{\text{spolIG}} \).

To examine the role of Spo0A binding sites in region I of \( P_{\text{spolIG}} \), a PCR mutagenesis protocol was used to alter the 0A box at -94 and insert two point mutations into the 0A box at -84 (Materials and Methods VI, 1). The mutant template (\( P_{\text{spolIG}} \) \text{M94/84}) was used in transcription assays to test the effect of Spo0A(-P) concentration on maximum \( P_{\text{spolIG}} \) transcription (Figure 29). A comparison of completion levels of transcription from \( P_{\text{spolIG}} \) \text{M94/84} and the wildtype promoter showed no obvious
Table IV. Effect of KAc concentration on rates of $P_{spoIIIG}$ transcription initiation in assays that contained various forms of the Spo0A protein.

KAc concentration was adjusted by adding various inputs of the salt to mixtures of $P_{spoIIIG}$ template, ATP, GTP and buffer, before incubating the DNA with phosphorelay samples. Initiation time-course reactions were begun by adding RNA polymerase and rate constants for heparin-resistant complex formation were measured using $k_{obs}$ plots (described in Figure 18). These value are shown below.

\[
\begin{array}{cccc}
\text{[KAc] (mM)} & \text{no Spo0A} & \text{Spo0A} & \text{Spo0A}^{BD} & \text{Spo0A-P} \\
0 & 0.469 & 1.19 & 1.17 & 2.95 \\
40 & 0.361 & 0.587 & 0.545 & 4.02 \\
80 & 0.344 & 0.421 & 0.201 & 4.31 \\
120 & 0.446 & 0.546 & 0.291 & 4.27 \\
\end{array}
\]
Figure 28. Kinetics of transcription initiation at $P_{spoII}$ at two KAc concentrations.

Numbers of $P_{spoII}$ transcripts in time samples of rate assays (described in Table II) that contained 0 (A) or 120 mM KAc (B), were calculated and plotted as a function of time. These assays contained no Spo0A (□), or 400 nM Spo0A (○), Spo0A$^{BD}$ (▲) or Spo0A-P (●). The lines drawn through each data set (except for A, Spo0A$^{BD}$) were calculated by linear least-squares analysis (coefficient constant > 0.9). Values derived from the slopes of these lines are given in Table VI.
A

![Graph A](image)

B

![Graph B](image)
Table V. Initial rates of transcript synthesis from $P_{spoIG} +/- 120$ mM KAc

The initial rates of transcript synthesis shown below were derived from the slopes on lines drawn through the data sets plotted in Figure 28. Assays contained either no KAc or 120 mM KAc and either no Spo0A or 400 nM Spo0A, Spo0A$^{BD}$, or Spo0A-P.

<table>
<thead>
<tr>
<th>[KAc] (mM)</th>
<th>initial rate (transcripts/sec x 10^-9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>no Spo0A (□)</td>
<td>0</td>
</tr>
<tr>
<td>Spo0A (○)</td>
<td>0</td>
</tr>
<tr>
<td>Spo0A$^{BD}$ (▲)</td>
<td>0</td>
</tr>
<tr>
<td>Spo0A-P (●)</td>
<td>0</td>
</tr>
<tr>
<td>no Spo0A (□), 120</td>
<td>120</td>
</tr>
<tr>
<td>Spo0A (○)</td>
<td>120</td>
</tr>
<tr>
<td>Spo0A$^{BD}$ (▲)</td>
<td>120</td>
</tr>
<tr>
<td>Spo0A-P (●)</td>
<td>120</td>
</tr>
</tbody>
</table>

* estimated from the initial and secondary slopes
Figure 29. Comparison of the effect of increased Spo0A(-P) concentration on wildtype and mutant $P_{spolIG}$ activity.

Transcription assays that contained serial dilutions of Spo0A (open symbols) or Spo0A-P (closed symbols) were carried out using either wildtype $P_{spolIG}$ (circles) or $P_{spolIG}^{M94/84}$ (triangles) as promoter template. To assay for completion levels of heparin-resistant complex formation, RNA polymerase and promoter template were incubated for 10 minutes prior to initiating elongation reactions. Promoter activity, defined as the percentage of $P_{spolIG}$ template transcribed, is plotted as a function of total Spo0A(-P) concentration.
Table VI. Comparison of $k_{\text{obs}}$ rate constants from transcription rate assays that contained wildtype or $P_{\text{spoIIG}}^{M94/84}$ promoter templates.

The effect of the mutations on the rate of initiation at $P_{\text{spoIIG}}$ was assessed in transcription rate assays that contained no Spo0A or with 200 nM Spo0A or Spo0A-P. Rate constants from assays performed on mutant and wildtype $P_{\text{spoIIG}}$ templates, were derived from $k_{\text{obs}}$ plots (not shown) and are shown below.

<table>
<thead>
<tr>
<th></th>
<th>$P_{\text{spoIIG}}^{M94/84}$</th>
<th>$P_{\text{spoIIG}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>no Spo0A</td>
<td>0.744</td>
<td>0.260</td>
</tr>
<tr>
<td>Spo0A</td>
<td>0.933</td>
<td>0.467</td>
</tr>
<tr>
<td>Spo0A-P</td>
<td>1.87</td>
<td>1.48</td>
</tr>
</tbody>
</table>
differences in stimulation by either Spo0A or Spo0A-P, although the mutant promoter appeared to be slightly more responsive to lower Spo0A-P concentrations. 

\(P_{spolIG}^{M94/84}\) was also tested in transcription rate assays that contained Spo0A(-P). The \(k_{obs}\) values measured for these assays are presented in Table VI and although slight differences were observed between the mutant and wildtype promoter in the absence of Spo0A or with 200 nM Spo0A, the disparities were within normal variation for rate assay experiments and were not considered to be significant.

Transcription experiments carried out on \(P_{spolIG}^{M94/84}\) revealed that mutations to the 0A boxes of region I did not significantly affect either the completion level or rate of transcription initiation at \(P_{spolIG}\). This suggests that the functional Spo0A binding sites in terms of transcription activation, are within region II of the promoter. However, because this hypothesis is based on only two experiments, further experimentation is required to eliminate the potential contribution of region I. In addition, the mutant promoter should be tested \textit{in vivo} to determine whether the normal \(P_{spolIG}\) transcription profile is affected.

V. DNaseI footprinting of Spo0A(-P) to \(P_{spolIG}\).

1. Previous reports of \(P_{spolIG}\) DNaseI protection studies.

It has been reported that Spo0A protects two regions of \(P_{spolIG}\) from DNaseI digestion (Satola \textit{et al.}, 1991). Each region contains two sequences with identity to canonical Spo0A binding sites (0A boxes). Region I contains 0A boxes centered at positions -94 and -84 relative to the transcription start-site, while the 0A boxes in region II are centered at -50 and -40 (Figure 2). Non-phosphorylated Spo0A appeared to bind both regions I and II although very high concentrations of Spo0A protein were required for DNaseI protection of region II (Satola \textit{et al.}, 1991). Based on this observation, it was proposed that region I contained high affinity Spo0A binding sites while region II contained low affinity binding sites. Baldus \textit{et al.} (1994) used either
acetyl phosphate or the heterologous sensor/kinase protein, NR II (Ninfa and Magasanik, 1986), to obtain a low level of Spo0A phosphorylation (approximately 1.0% of the protein). They observed that phosphorylation of Spo0A increased DNaseI protection to all OA boxes at $P_{spolIG}$, but appeared to enhance Spo0A binding to region II in particular.

2. Effect of increased Spo0A(-P) concentration on DNaseI protection of $P_{spolIG}$.

Previous $P_{spolIG}$ footprinting studies were conducted with Spo0A which had been phosphorylated to a very low level. Therefore, the effect of phosphorylation on Spo0A footprinting at $P_{spolIG}$ was reexamined using the phosphorelay reaction to obtain high levels of Spo0A-P. Figure 30 shows DNaseI footprints produced by increasing concentrations of Spo0A(-P). Binding of non-phosphorylated Spo0A to the promoter appeared to be weak. Only slight protection to OA boxes in region I was detected at the highest concentrations of protein. In contrast, concentrations of 200 - 400 nM Spo0A-P protected both regions of the promoter producing footprints that extended from -105 to -80 in region I and from -60 to -35 in region II. This suggested that both OA boxes in each region were bound by Spo0A-P. The DNA between these two areas of protection was naturally resistant to DNaseI cleavage (see control lane), so it is not known whether Spo0A-P also bound to this region of the promoter.


The transcription initiation model (R11) (Results IV, 8), suggests there may be multiple types of heparin-sensitive polymerase/$P_{spolIG}$ complexes on the pathway to heparin-resistant complex formation. Since transcription rate assays containing Spo0A-P required approximately one minute to reach maximum heparin-resistant complex formation, and reactions containing Spo0A or no Spo0A took even longer, it was anticipated that structural changes between different heparin-sensitive complexes might be detected by DNaseI protection assays. Therefore, footprinting experiments were conducted on samples removed from RNA polymerase/$P_{spolIG}$ time-course binding
Figure 30 The effect of Spo0A(-P) concentration on DNaseI footprints in the absence of RNA polymerase.

DNaseI footprint reactions were carried out by incubating $P_{spoIIG}$ fragment (non-transcribed strand labeled) with various concentrations of Spo0A or Spo0A-P (lane 1, 50 nM; lane 2, 100 nM; lane 3, 200 nM; lane 4, 400 nM) for five minutes before digesting with DNaseI. Lane C is a control DNaseI footprint of $P_{spoIIG}$ with no added protein. The nucleotide positions indicated are relative to the transcription start-site.
reactions. GTP was omitted from these reactions to prevent the polymerase from reaching a heparin-resistant state (Results 1, 2). RNA polymerase binding reactions were initiated by adding polymerase to mixtures of $P_{spoIIG}$ template and either no Spo0A, or 400 nM Spo0A(-P). At various times, samples were removed and digested with DNaseI for 10 seconds. Protection patterns resulting from experiments with $P_{spoIIG}$ template, labeled on either the non-transcribed or transcribed DNA strands, suggested that distinctly different complexes were formed under the various conditions (Figures 31 and 32).

In time-course assays that contained no Spo0A protein, a RNA polymerase footprint was clearly visible in the first (5 second) sample and did not appear to change in subsequent time-points. This suggested that complex formation between the polymerase and $P_{spoIIG}$ (designated as C1) was virtually complete within 15 seconds (5 seconds plus 10 second digest). The C1 footprint was characterized by DNaseI protection extending from positions -60 to -24 on the non-transcribed strand (Figure 31) and -65 to -20 on the transcribed strand (Figure 32). In addition, the region between -20 and +10 on the transcribed strand appeared to be weakly protected. Conspicuous DNaseI hypersensitive sites were evident at -45 and -23 on the non-transcribed strand. Position -55, which was readily cleaved in control DNaseI digestions, was also unprotected by the C1 complex. Interestingly, protection of the transcribed strand was less evident in the two minute time sample, and by 15 minutes there was essentially no protection to either DNA strand. Therefore, it was assumed that binding of the polymerase to $P_{spoIIG}$ had been reversible. Our lab has observed that free RNA polymerase in solution is quickly inactivated at 37° C. Thus, the combined effects of a high rate of dissociation and polymerase inactivation may account for loss of the C1 footprint over time.

DNaseI footprint experiments carried out on time-course binding reactions that contained RNA polymerase and non-phosphorylated Spo0A exhibited two distinct DNaseI protection patterns. The footprint detected in the 5 second sample was very
Figure 31 Kinetic DNaseI footprints at $P_{\text{spoII}G}$ (non-transcribed strand).

Influence of Spo0A and Spo0A-P on RNA polymerase complexes formed at $P_{\text{spoII}G}$. Time-course DNaseI protection assays were carried out with reactions that contained RNA polymerase and either no Spo0A, 400 nM Spo0A, or 400 nM Spo0A-P. After the addition of RNA polymerase (5 sec, lane 1; 30 sec, lane 2; 1 min, lane 3; 2 min, lane 4; or 5 min, lane 5), samples were removed and subjected to a 10 second DNaseI digestion. Lane C is a control DNaseI digestion of $P_{\text{spoII}G}$ in the absence of protein. Lane 0 in 'no Spo0A', 'Spo0A', and 'Spo0A-P' panels contained no protein, Spo0A only, or Spo0A-P only, respectively. The indicated nucleotide positions are relative to the transcription start-site (arrow).
Figure 32 Kinetic DNaseI footprint at $P_{spoIIG}$ (transcribed strand).

Time-course DNaseI protection assays were carried out with reactions that contained RNA polymerase (100 nM) and either no Spo0A, 400 nM Spo0A, or 400 nM Spo0A-P, as described in Figure 31. After the addition of RNA polymerase (5 sec, lane 1; 30 sec lane 2; 1 min, lane 3; 1.5 min, lane 4; 2 min, lane 5; and 15 min, lane 6). Lane C is a control DNaseI digestion of $P_{spoIIG}$ fragment in the absence of protein. Lane 0 in 'no Spo0A', 'Spo0A' and 'Spo0A-P' panels contained no protein, Spo0A only, or Spo0A-P only, respectively. The nucleotide positions indicated are relative to the transcription start-site (arrow).
similar to the C_I footprint, based on the appearance of a -45 DNaseI hypersensitive site on the non-transcribed strand. However, the -45 position was protected in later samples suggesting that C_I was replaced by a new complex (C_{II}). C_{II} was identified by expanded limits of DNaseI protection. The footprint extended downstream toward -10, and upstream to encompass Spo0A binding sites centered at -84 and -94. In addition, the -45 and -23 DNaseI hypersensitive sites of the C_I footprint were replaced by new hypersensitive sites at positions -28 and -27 on the non-transcribed strand, and positions -28 and -26 on the transcribed strand. A gradual increase in the intensity of these hypersensitive sites in later time-course samples, indicated that formation of the C_{II} complex occurred slowly compared to C_I. Since the C_{II} footprint was observed only in DNaseI assays that contained RNA polymerase and Spo0A, it was assumed to result from a Spo0A/ polymerase complex.

Control samples removed from binding reactions that contained Spo0A only (Figure 31), resulted in weak DNaseI protection to the 0A boxes of region I. Protection to this region became more distinct when RNA polymerase was added to the reaction, indicating that a synergistic interaction occurred between Spo0A and the polymerase. It appeared that RNA polymerase had bound to the promoter and then facilitated binding of Spo0A to the 0A boxes in region I. It could not be determined with certainty whether Spo0A had also bound to either of the 0A boxes in region II because DNaseI protection provided by RNA polymerase alone overlapped this portion of the promoter (C_I footprint, Figures 31 and 32). It is interesting to note that the -45 hypersensitive site and the major unprotected site at -55 on the non-transcribed strand of a C_I footprint (Figure 31), were protected from DNaseI cleavage in the C_{II} footprint. Since these hypersensitive sites are located on either side of the 0A box at -50, it is possible that protection may have resulted from Spo0A binding to this site after polymerase had bound $P_{spoIIG}$. Binding of Spo0A to the 0A box at -50 may also have caused the gradual transition from C_I to C_{II} coinciding with the appearance of hypersensitivity to
the -28, -27 and -26 positions. It is possible that hypersensitivity within this region of the promoter resulted from a distortion of the DNA caused by a Spo0A dependent isomerization in polymerase structure.

Despite the apparent cooperative interaction between RNA polymerase and Spo0A, C II complexes appeared to be only slightly more stable than C I. Although the C II footprint was distinct in the two minute time-point of Figure 32, it had disappeared from both DNA strands by the 15 minute time-point, suggesting that like C I, C II had a high rate of dissociation leading to polymerase inactivation.

Addition of Spo0A-P to time-course binding reactions resulted in the formation of a third complex (C III). The C III footprint covered essentially the same region of PspoIIG as did C II. A loss of the DNaseI hypersensitive sites unique to the C II footprint was a distinguishing feature of the C III protection pattern. Although the -28 hypersensitive site on the transcribed strand was retained in a C III footprint, the -26 site disappeared, as did both the -28 and -27 sites of the non-transcribed strand. The control lane which shows the protection pattern resulting from Spo0A-P only, indicated the protein bound to regions I and II of PspoIIG. Unlike the reaction that contained Spo0A, no transient C I footprint was observed, suggesting C III formation was very rapid. As in the case of C II, it was assumed that protection upstream of -80 in the C III footprint resulted from Spo0A-P binding to region I. While there was no definitive evidence to indicate whether the protein also bound to the 0A boxes of region II, improved DNaseI protection between positions -62 and -32 of the transcribed strand implied that Spo0A-P binding to both 0A boxes in this region may have been an integral part of the C III complex. It is possible that the loss of hypersensitivity in the -28 to -26 region of both strands of the promoter could indicate that the DNA distortion evident in C II was resolved in the transition to C III either because of Spo0A-P binding or an additional/alternative isomerization within the polymerase.
An additional difference between CIII and the C1 or CII complexes was the apparent stability of CIII. The CIII protection pattern was clearly visible even in the 15 minute time-point samples from reactions containing Spo0A-P. Thus, CIII appeared to have a reduced rate of dissociation, due either to the influence of Spo0A-P binding to a particular site, or because Spo0A-P had induced the polymerase to isomerize into a complex with greater stability.

4. The effect of temperature on kinetic DNaseI footprints.

In vitro assays showed PspoIIIG transcription from linearized template was extremely temperature sensitive (Results III, 1). To examine the possibility that transitions between C1, CII and CIII might be affected by lower temperature, time-course DNaseI protection assays carried out on the non-transcribed strand of PspoIIIG at 15°, 25°, and 31° C. The footprints obtained with RNA polymerase and either no Spo0A, Spo0A, or Spo0A-P indicated that lower temperatures had a significant effect on complex transitions (Figures 33 - 35).

DNaseI protection patterns obtained at 25° and 31° C with RNA polymerase only, suggested that formation of the C1 complex was no different than at 37° C. Binding appeared to be complete by the first time sample and the footprint exhibited the same pattern of DNaseI hypersensitivity. Reactions that contained polymerase and Spo0A-P produced footprints at 25° and 31° C that were virtually identical to the CIII footprint obtained at 37° C. Therefore, it appeared that CIII formation was also unaffected by the lower temperatures.

In contrast, the reaction containing polymerase and non-phosphorylated Spo0A produced DNaseI protection patterns that indicated CII formation had been adversely affected by lower temperatures. Judging by the relative intensities of the -28 and -27 DNaseI hypersensitive sites, both the maximum level and the rate of CII formation were reduced, especially at 25° C. Although the first time samples at both temperatures produced protection patterns that appeared to be similar to the CIV/CII composite
obtained at 37°C, the transition to the CII type footprint in later time samples was
slower. However, the rapid disappearance of the -23 and -45 hypersensitive sites and
increased protection extending to the -10 position, did not appear to parallel the delayed
emergence of the CII footprint. This suggested the presence of an additional step
between CI and CII which was characterized by protection to positions -23 and -45 and
extension of the polymerase footprint to the -10 domain, but produced no
hypersensitivity to the -28 and -27 positions. The changing DNaseI protection patterns
of the Spo0A reaction were interpreted as evidence that the binding of polymerase and
Spo0A to the promoter had not been affected by reduced temperature, but that a
subsequent isomerization step had been impaired.

Temperature sensitivity of the transition between CI and CII was clearly evident in
the DNaseI protection experiment carried out at 15°C (Figure 35). Appearance of the
-45 and -23 hypersensitive sites indicated that the polymerase formed a normal CI
complex in the absence of Spo0A protein. However, the low temperature completely
blocked development of -28 and -27 hypersensitive sites that characterized footprints
obtained from reactions that contained Spo0A. If these sites are indicators for an
isomerization in polymerase conformation, it would seem that this step was completely
inhibited at 15°C, even though protection to region I of the promoter showed that
Spo0A protein binding appeared to be unaffected. Because the CIII footprint has no
distinguishing hypersensitive sites on the non-transcribed strand, it is difficult to assess
low temperature effects on its formation. However, similarities between the footprints
obtained with Spo0A and Spo0A-P at 15°C suggested that CIII formation was also
blocked despite a DNaseI protection pattern that showed the binding of polymerase and
Spo0A-P to be unchanged.

Two additional differences between footprints obtained at 37°C and the lower
temperatures should be noted. First, the stability of the CI and CII complexes seemed to
increase substantially at 31°C and 25°C, since these footprints were still clearly visible in
Figure 33  Kinetic DNaseI footprint at 31° C.

Time-course DNaseI protection assays were carried out as described in Figure 31 except that the temperature was 31° C. After the addition of 100 nM RNA polymerase (5 sec, lane 1; 30 sec, lane 2; 90 sec, lane 3; and 10 min, lane 4), samples were removed and subjected to a 10 second DNaseI digestion. Lane C is a control DNaseI digestion of \( P_{spoIIIG} \) fragment (non-transcribed strand labeled) in the absence of protein. Lane 0 in 'Spo0A', or 'Spo0A-P' panels contained no protein, Spo0A only, or Spo0A-P only, respectively.
Figure 34 Kinetic DNaseI footprint at 25°C.

Time-course DNaseI protection assays were carried out as described in Figure 31 except that the temperature was 25°C. After the addition of 100 nM RNA polymerase (5 sec, lane 1; 30 sec, lane 2; 90 sec, lane 3; and 10 min, lane 4), samples were removed and subjected to a 10 second DNaseI digestion. Lane C is a control DNaseI digestion of P_{spoIIG} fragment (non-transcribed strand labeled) in the absence of protein. Lane 0 in 'Spo0A', or 'Spo0A-P' panels contained no protein, Spo0A only, or Spo0A-P only, respectively. The nucleotide positions indicated are relative to the transcription start-site (arrow).
Figure 35 Kinetic DNaseI footprint at 15° C.

Time-course DNaseI protection assays were carried out as described in Figure 31 except that the temperature was 15° C. After the addition of 100 nM RNA polymerase (5 sec, lane 1; 30 sec, lane 2; 90 sec, lane 3; and 10 min, lane 4), samples were removed and subjected to a 10 second DNaseI digestion. Lane C is a control DNaseI digestion of $P_{spoIIG}$ fragment (non-transcribed strand labeled) in the absence of protein. Lane 0 in 'no Spo0A', 'Spo0A', or 'Spo0A-P' panels contained no protein, Spo0A only, or Spo0A-P only, respectively. The nucleotide positions indicated are relative to the transcription start-site.
the 10 minute time-point samples. Lower temperatures may have reduced the
dissociation rate of polymerase/P_{spoII}G complexes which was believed to contribute to
a rapid inactivation of the enzyme. Secondly, a more thorough protection from DNaseI
cleavage was observed in the -20 to -10 region of C_{II} and C_{III} footprints. This was
particularly noticeable for positions -17 and -13 which had been poorly protected in
footprint assays conducted at 37° C. It is possible that lower temperature stabilized an
interaction between the polymerase and this region of the promoter that may have
occurred only transiently at the higher temperature.

5. Effect of SpoOA(-P) concentration on RNA polymerase complexes at P_{spoII}G.

Kinetic DNaseI protection assays identified at least three distinct heparin-sensitive
complexes formed between RNA polymerase and P_{spoII}G. Formation of the C_{II} and C_{III}
complexes was clearly dependent on SpoOA or SpoOA-P. Therefore, it was decided to
investigate how relative levels of each type of complex, might be affected by changes in
SpoOA(-P) concentration. The experiment was carried out by incubating P_{spoII}G
template (labeled on the non-transcribed strand) with serial dilutions of phosphorelay
samples containing SpoOA or SpoOA-P at 37° C (Figure 36).

At the lowest input of non-phosphorylated SpoOA (25 nM), a composite of C_{I} and
C_{II} footprints was indicated by the presence of hypersensitive sites at positions -45/-23
(C_{I}) and -28/-27 (C_{II}). As judged by the diminished intensity of the -45 and -23
hypersensitive sites, and greater prominence of the -28 and -27 sites, relative levels of
each complex shifted toward C_{II} as the concentration of SpoOA was increased. No
evidence of a C_{I} footprint was observed at SpoOA concentrations above 200 nM.

The DNaseI footprint observed at 25 nM SpoOA-P exhibited a thorough protection of
an extensive portion of the promoter indicative of C_{III} formation. No DNaseI
hypersensitivity was detected at positions -45 or -23. Therefore, none of the complexes
appear to have been C_{I}. However, faint -28 and -27 DNaseI hypersensitive sites were
taken as evidence that a few C_{II} had been formed. Because the C_{III} footprint has no
Figure 36 Effect of Spo0A(-P) concentration on $P_{spoIIG}$ DNaseI footprints.

DNaseI footprint reactions were carried out by incubating $P_{spoIIG}$ fragment (non-transcribed strand labeled) with various concentrations of Spo0A or Spo0A-P (no Spo0A(-P), lane 0; 25 nM, lane 1; 50 nM, lane 2; 100 nM, lane 3; 200 nM, lane 4; 400 nM, lane 5; and 800 nM, lane 6) and a fixed concentration of RNA polymerase (25 nM) for five minutes before digesting with DNaseI. Lane C is a control DNaseI footprint of the $P_{spoIIG}$ fragment with no added protein.
distinguishing sites of DNaseI hypersensitivity, the remainder of the complexes formed at low Spo0A-P concentration were probably CIII but could not be detected. Loss of the -28 and -27 DNaseI hypersensitive sites with increased concentrations of Spo0A-P, indicated that no CII complexes were formed at Spo0A-P concentrations of 100 nM and above.

6. DNaseI footprinting of $P_{spoIIG}^{M94/84}$.

Since the Spo0A binding sites within region I of $P_{spoIIG}$ are approximately 15 - 20 bps upstream of DNA sequences that were protected by RNA polymerase alone (see C1 complex, Results V, 2 & 3) it is not likely that Spo0A(-P) protein bound to these 0A boxes would be able to contact the polymerase. Therefore, it was unclear how Spo0A(-P) bound to region I of the promoter contributed to $P_{spoIIG}$ transcriptional activity. To investigate a possible role for the Spo0A binding sites of region I, DNaseI protection experiments were carried out using the mutant construct, $P_{spoIIG}^{M94/84}$ (Materials and Methods VI, 1 & IV, 11).

Lack of DNaseI protection to the upstream portion of $P_{spoIIG}^{M94/84}$ suggested the mutations had abolished binding of Spo0A(-P) to the 0A boxes of region I. In addition, no DNaseI protection of region II of the promoter was detected in the reactions that contained Spo0A only (Figure 37). Addition of Spo0A-P resulted in DNaseI protection to 0A boxes at -50 and -40 but the level of protection was reduced compared to similar protein concentrations on the wildtype promoter. DNaseI protection assays that contained Spo0A(-P) and RNA polymerase (Figure 38) resulted in C1, CII and CIII type footprints, however, relative intensities of the -45/-23 (C1) and -28/-27 (CII) hypersensitive sites indicated that the transition from C1 to CII required higher concentrations of Spo0A than the wildtype promoter. Moreover, binding reactions that contained Spo0A-P exhibited DNaseI hypersensitive sites unique to both C1 and CII, suggesting a higher concentration of Spo0A-P was also required to obtain a CIII footprint.
Figure 37 Effect of increased Spo0A(-P) concentration on DNaseI footprinting at $P_{\text{spolIG}^\text{M94/84}}$.

Effect of Spo0A(-P) concentration of RNA polymerase complexes formed at $P_{\text{spolIG}^\text{M94/84}}$. DNaseI footprint reactions were carried out by incubating $P_{\text{spolIG}^\text{M94/84}}$ fragment (non-transcribed strand labeled) with various concentrations of Spo0A or Spo0A-P (50 nM, lane 1; 100 nM, lane 2; 200 nM, lane 3; 400 nM, lane 4) and a fixed concentration of RNA polymerase for five minutes before digesting with DNaseI. Lane C is a control DNaseI footprinting of $P_{\text{spolIG}^\text{M94/84}}$ with no added protein. Nucleotide positions indicated are relative to the transcription start-site.
Figure 38 Effect of Spo0A(-P) concentration on \( P_{\text{spoII}} \)\(^{M94/84}\) footprints at constant RNA polymerase concentration.

Effect of Spo0A(-P) concentration of RNA polymerase complexes formed at \( P_{\text{spoII}} \)\(^{M94/84}\). DNaseI footprint reactions were carried out by incubating \( P_{\text{spoII}} \)\(^{M94/84}\) fragment (non-transcribed strand labeled) with various concentrations of Spo0A or Spo0A-P (no Spo0A(-P), lane 0; 25 nM, lane 1; 50 nM, lane 2; 100 nM, lane 3; 200 nM, lane 4; and 400 nM, lane 5) and a fixed concentration of RNA polymerase (100 nM) for five minutes before digesting with DNaseI. Lane C is a control DNaseI footprint of the \( P_{\text{spoII}} \)\(^{M94/84}\) fragment with no added protein. Nucleotide positions indicated are relative to the transcription start-site (arrow).
These results are consistent with the hypothesis that Spo0A(-P) bound to the 0A boxes of region I might promote binding of the activator to the 0A boxes of region II through a cooperative interaction. More significantly, the footprinting experiments performed on $P_{spoIIG}^{M94/84}$ demonstrated that the 0A boxes in region II were the functionally important sites for catalyzing polymerase isomerizations resulting in C$_{II}$ and C$_{III}$ formation.

VI. Investigation of $P_{spoIIA}$ transcription stimulation by Spo0A-P.

1. $P_{spoIIA}$ transcription assays.

*In vitro* experiments had established that expression of the *spoIIA* operon was dependent on the alternative sigma factor, $\sigma^H$ (Wu *et al.*, 1991). Therefore, $P_{spoIIA}$ transcription assays were carried out with either core RNA polymerase or $\sigma^A$ holoenzyme, mixed with recombinant $\sigma^H$ protein. These experiments were carried out with a plasmid construct obtained by cloning $P_{spoIIA}$ into a pUC18 based plasmid (pPS-28) which carried tandem (T$_1$T$_2$) transcription terminators isolated from the rrnB rRNA operon of *E. coli* (Materials and Methods II, 1). This resulted in a $P_{spoIIA}$ template which could be assayed as either relaxed or supercoiled DNA.

Three major RNA products of different sizes were produced in $P_{spoIIA}$ transcription assays (Figure 39). Synthesis of the two smaller transcripts (transcripts II and III) was dependent on $\sigma^H$, while the largest transcript (transcript I) was apparently transcribed by RNA polymerase containing $\sigma^A$. Primer extension analysis of transcripts II and III, demonstrated that both originated from $P_{spoIIA}$ and that the *in vitro* start-site was identical to that reported for *spoIIA* mRNA produced *in vivo* (Figure 40). It was assumed that read through of the T$_1$ transcription terminator accounted for the difference in size between transcripts II and III. Because no RNA products were synthesized in transcription assays performed on the pPS-28 vector, it appeared that transcript I originated from the cloned *B. subtilis* DNA fragment which carried $P_{spoIIA}$. 
However, no primer extension product was obtained when this RNA was used as a template for reverse transcriptase and the exact origin of the transcript was not determined.

Two characteristics of $P_{\text{spoIIA}}$ transcriptional activity were found to differ from those of $P_{\text{spoIIG}}$. First, significant levels of transcripts were produced by $P_{\text{spoIIA}}$ only if the promoter was assayed as a supercoiled template (Bird et al., 1992). Secondly, the formation of heparin-resistant complexes at $P_{\text{spoIIA}}$ did not require initiating NTPs. These properties were interpreted as evidence that the mechanisms of transcription initiation at $P_{\text{spoIIA}}$ and $P_{\text{spoIIG}}$ were different.

2. The effect of Spo0A-P on the extent and rate of $P_{\text{spoIIA}}$ transcription initiation.

To determine whether Spo0A(-P) could affect $P_{\text{spoIIA}}$ activity, aliquots from equilibrated phosphorelay reactions were added to transcription assays using the same procedure as for $P_{\text{spoIIG}}$ (Results I, 1). These experiments indicated that $P_{\text{spoIIA}}$ activity was stimulated by Spo0A-P (Figure 41). This effect was further examined by adding serial dilutions of kinase reaction samples to transcription assays to determine the effect of various Spo0A(-P) concentrations on $P_{\text{spoIIA}}$ activity. While concentrations of up to 700 nM Spo0A-P continued to enhance transcription from $P_{\text{spoIIA}}$, similar inputs of non-phosphorylated Spo0A or mixtures of phosphorelay components which lacked Spo0A, had no influence on $P_{\text{spoIIA}}$ activity.

The possibility that Spo0A-P might stimulate the rate of transcription initiation at $P_{\text{spoIIA}}$ was investigated by adding phosphorelay samples to time-course transcription assays that contained 480 nM Spo0A(-P) (Figure 41B). In each case, maximum transcription was reached within five minutes, and although Spo0A-P stimulated the maximum level of transcription compared to Spo0A, the rate of approach to the respective maxima appeared to be similar. A preliminary investigation of the initiation process at $P_{\text{spoIIA}}$ indicated that initiation rates were too fast to measure
Radioactive products from \textit{in vitro} transcription of the plasmid pIIA-28 were separated on a 5\% polyacrylamide gel and detected by autoradiography. Three major transcript bands were observed and are designated I, II or III. The promoter template was assayed as supercoiled plasmid (lanes 1, 3, 4, 6, 7 and 9) or as linearized DNA following a HindIII restriction digest (lanes 2, 5 and 8). The assays were carried out by adding both core RNA polymerase and $\sigma_H$ (lanes 1, 2, 4, 5, 7 and 8) or core polymerase only (lanes 2, 5 and 8) to each reaction (Materials and Methods IV, 6). No Spo0A protein was added to reactions shown in lanes 1 - 3. Lanes 4 - 5 contained samples from phosphorelay protein mixtures that lacked KinA and ATP (Spo0A), while lanes 6 - 9 contained samples from equilibrated phosphorelay reactions (Spo0A-P). The final Spo0A concentration in transcription assays that received phosphorelay samples was 480 nM.

Figure 39. \textit{In vitro} transcription products of $P_{\text{spoIIA}}$. 
Figure 40. Primer extension analysis of $P_{spolA}$ transcripts.

Transcripts II and III generated from spoIIA transcription assays (see Figure 39) were electroeluted from polyacrylamide gel slices, and the purified RNA was used as template for primer extension (Materials and Methods IV, 7). The same primer used for extension reactions was used to produce the DNA sequence of the $P_{spolA}$ region. The DNA sequence in the region of the in vivo transcription start-site (●) is shown to the left, and the primer extension product is indicated by the arrow.
Figure 41.A. Stimulation of $P_{spolIA}$ transcription by increasing Spo0A-P concentration.

Transcription assays contained dilutions of phosphorelay reaction/protein mixtures to yield the indicated Spo0A protein concentrations. The kinase reactions contained all components (-●-), or all components except Spo0A (-□-), or all components except KinA and ATP (-○-). Transcription products were separated by electrophoresis and quantitated by measuring Cerenkov radiation in gel slices containing transcript III. In the case of transcription reactions containing samples of the phosphorelay mixture which did not include Spo0A, the data is plotted according to the dilution of kinase reaction samples that did contain Spo0A protein.

Figure 41.B. The effect of Spo0A-P on the rate of transcription initiation at $P_{spolIA}$.

(B) Time-course reactions were initiated by adding core RNA polymerase to a mix of pIIA-28 (4.8 nM) $\sigma^H$ protein (480 nM) and either 480 nM Spo0A (-○-) or Spo0A-P (-●-). At the indicated times, aliquots were removed and transferred to tubes containing heparin (5 µg/mL final concentration). After a five minute incubation in the presence of heparin, elongation of RNA was permitted by addition of a mix containing all four nucleotide triphosphates. Transcription products were separated by electrophoresis and quantitated by determining the Cerenkov radiation in the gel slices containing labeled transcript III.
Figure 42. Effect of increasing $\sigma^H$ protein concentration on $P_{spolIIA}$ activity.

Transcription assays were carried out with various dilutions of $\sigma^H$ protein and a constant amount of core RNA polymerase. The final $\sigma^H$ concentrations in these assays were: lane 1, 0.1 $\mu$M; lane 2, 0.2 $\mu$M; lane 3, 0.4 $\mu$M; lane 4, 0.8 $\mu$M; lane 5, 1.6 $\mu$M. Since there is no direct assay to determine the specific activity of recombinant $\sigma^H$ protein, these concentrations reflect the final concentrations of total protein. Reactions were conducted in the presence or absence of phosphorylation reaction samples (+/- 480 nM Spo0A-P). The RNA products from these assays were separated by electrophoresis and visualized by autoradiography (A). $P_{spolIIA}$ activity was quantitated by measuring Cerenkov radiation in gel slices containing transcript III and are plotted as a function of total $\sigma^H$ protein (B) in the absence (-○-) or presence (-●-) of Spo0A-P.
A  

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<tr>
<th>no Spo0A</th>
<th>Spo0A-P</th>
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<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
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B  

![Graph showing the effect of [Spo0H] on P_{Spo0A} activity](image)
through transcription rate assays, therefore, a kinetic analysis of this promoter was not pursued.

3. The combined effect of Spo0A-P and $\sigma^H$ concentration on $P_{spoIIA}$ activity.

*In vivo* levels of active $\sigma^H$ protein are known to rise at about the same time the expression of the *spoIIA* operon begins. Therefore, the expression of the *spoIIA* operon is potentially dependent on both Spo0A phosphorylation and the intracellular concentration of $\sigma^H$ protein. The effect of $\sigma^H$ concentration on transcription from $P_{spoIIA}$ was examined by adding various dilutions of the protein to $P_{spoIIA}$ transcription assays that contained no Spo0A or a constant amount of Spo0A-P (Figure 42). As expected, promoter activity was very responsive to increased $\sigma^H$ concentration, but this was especially true for transcription assays that contained Spo0A-P. It was estimated that transcription assays without Spo0A-P required three times the concentration of $\sigma^H$ to reach the same level of $P_{spoIIA}$ transcription as in reactions containing Spo0A-P.
Discussion

I. Summary of results.


Transcription assays clearly demonstrated that purified Spo0A stimulated the transcription from $P_{spolIG}$ and $P_{spolIA}$, particularly after the protein had been modified by the phosphorelay. Phosphorylation of Spo0A increased maximum promoter stimulation and substantially lowered the concentration of Spo0A required to reach maximum activity at both $P_{spolIG}$ (Results II, 3) and $P_{spolIA}$ (Results VI, 2). The effect of phosphorylation on the ability of the Spo0A to stimulate transcription was clearly demonstrated by coupling several $P_{spolIG}$ transcription assays to a phosphorelay time-course reaction (Results II, 5). Enhanced stimulation of $P_{spolIG}$ closely paralleled progression of the kinase reaction and thus demonstrated the potential of the phosphorelay to function as a signal transduction system regulating Spo0A activity. This was taken as corroborative evidence for the hypothesis that Spo0A and the phosphorelay system are directly responsible for activation of the $spolIG$ and $spolIA$ operons in vivo. Activation of these operons which encode the developmental sigma factors $\sigma^E$ and $\sigma^F$, represents a crucial step in the commitment to cellular differentiation and testifies to the central role of Spo0A and the phosphorelay in the induction of sporogenesis.

2. Summary of the investigation of Spo0A(-P) stimulation of $P_{spolIG}$.

Transcription rate assays provided an effective means of investigating the mechanism through which Spo0A(-P) stimulated $P_{spolIG}$ activity. These experiments indicated that Spo0A-P catalyzed a rate limiting step in a transcription initiation reaction that was unaffected by the concentrations of the two reactants, RNA polymerase and $P_{spolIG}$ template (Results IV, 4, 5, & 7). This suggested that Spo0A-P influenced an
isomerization step that followed a rapid equilibration of RNA polymerase binding to the promoter.

DNaseI protection assays showed that phosphorylation of Spo0A significantly enhanced its ability to bind $P_{spolIG}$ especially to 0A boxes situated at the -50 and -40 positions (region II). In addition, these experiments provided compelling visual evidence that RNA polymerase did not require assistance from Spo0A(-P) to bind $P_{spolIG}$. Instead, Spo0A(-P) dependent transitions in polymerase/$P_{spolIG}$ footprints indicated that the transcription factor affected structural transformations in ternary complexes formed at the promoter.

II. Interpretation of in vitro transcription and DNaseI footprinting data.

1. Correlation between $P_{spolIG}$ transcription assays and DNaseI footprinting experiments.

Collectively, DNaseI footprinting experiments carried out on $P_{spolIG}$ compared well with the kinetic data from transcription rate assays. DNaseI protection patterns indicated that relative levels of $C_I$, $C_{II}$, and $C_{III}$ complexes were dependent on the amount of Spo0A or Spo0A-P added to footprint reactions (Results V, 5). Moreover, the formation of each type of complex appeared to correlate with different levels of $P_{spolIG}$ activity in transcription assays that contained similar Spo0A(-P) concentrations. In the absence of Spo0A(-P), kinetic footprinting assays indicated that $C_I$ formation was very rapid (Figure 31 & 32). But transcription rate assays that contained no Spo0A suggested that $C_I$ was equated with a slow rate of initiation and low completion levels of $P_{spolIG}$ transcription. A transition from $C_I$ to $C_{II}$ was observed footprint assays that contained high concentrations of non-phosphorylated Spo0A or very low concentrations of Spo0A-P. This was associated with a modest rise in both the rate of initiation and completion levels of activity in transcription rate assays that contained similar Spo0A(-P) concentration. Maximum stimulation to the rate and completion level of $P_{spolIG}$
transcription correlated with the formation of a C_{III} footprint, but was observed only in transcription or DNaseI protection assays that contained at least 100 - 200 nM Spo0A-P.

2. Interpretation of *P_{spoIIG}* transcription kinetics and DNaseI footprints.

Although transcription rate assays measured the kinetics of heparin-resistant complex formation (Results IV, 1 & 2), DNaseI footprinting experiments were carried out in the absence of GTP. Thus, C_{I}, C_{II}, and C_{III} must all be heparin-sensitive complexes that precede the heparin-resistant state. In reconsidering the reaction model (R11) discussed earlier (Results IV, 8);

\[
    R + P \xrightarrow{k_1/k_{-1}} HS_1 + 0A(-P) \xrightarrow{k_2/k_{-2}} HS_2 \xrightarrow{k_f/k_{fr}} HR
\]

it is apparent that C_{I} is equivalent to HS_{1}. Therefore, C_{II} and C_{III} must represent HS reaction intermediates resulting from isomerization steps toward HR. It is possible that C_{II} and C_{III} may be intermediates from separate initiation pathways that are dependent on the action of either Spo0A or Spo0A-P, or they may represent consecutive intermediates of the transcription initiation process at *P_{spoIIG}*. From the data obtained in transcription and footprinting experiments, it could not be determined which of these alternatives best describes the reaction pathway through which Spo0A(-P) stimulated *P_{spoIIG}* transcription.

From the changing patterns of DNaseI hypersensitivity in footprinting assays that contained RNA polymerase and various concentrations of Spo0A or Spo0A-P, it was apparent that heterogeneous populations of ternary complexes were formed at the promoter. Moreover, the observed transitions between different footprint patterns under these conditions suggested that Spo0A(-P) influenced equilibrium levels of C_{I}, C_{II} and C_{III} complexes. Thus, the effect of Spo0A and Spo0A-P on the relative distribution of various ternary complexes provides a rationale for understanding how transcription activation of *P_{spoIIG}* is achieved. In the absence of Spo0A(-P), RNA polymerase binds
rapidly to $P_{spoIIG}$ indicating the forward rate constant, $k_1$, is very large. But since few complexes are able to isomerize past $C_I$, transcription levels remain low. Addition of Spo0A or Spo0A-P drives the initiation reaction in the forward direction because they affect individual rate constants causing equilibrium levels of ternary complexes to shift toward the $C_{II}$ or $C_{III}$ intermediates. Consequently, the maximum level of promoter activity and rate of productive transcription initiation at $P_{spoIIG}$, is stimulated. The kinetic analysis could not distinguish whether Spo0A(-P) functioned to increase a forward rate constant ($k_2$) or decrease a reverse rate constant ($k_2$), however, either influence would have the effect of raising the concentration of intermediate ($C_{II}$ or $C_{III}$) complexes.

Comparison of the rapid appearance of DNaseI footprints with the slow rates of HR formation in rate assays that contained no Spo0A or non-phosphorylated Spo0A, showed that $C_I$ formation occurred prior to the rate limiting step in the initiation reaction. In contrast, the Spo0A-P dependent formation of $C_{III}$ was associated with very high rates of transcription initiation. Although $C_{III}$ is not heparin-resistant, it must be capable of a very rapid conversion to HR suggesting that Spo0A-P catalyzes a rate limiting initiation step by facilitating $C_{III}$ formation. $C_{III}$ may be analogous to a transition state intermediate which has achieved the activation energy required for productive transcription initiation. Spo0A-P could act as a catalyst for transcription initiation by lowering the activation energy of $C_{III}$ formation. This would effectively increase the concentration of $C_{III}$ complexes and thus cause the rate of transcription initiation to accelerate.

III. A model for transcriptional stimulation of $P_{spoIIG}$.

1. Compensating for the unusual structure of $P_{spoIIG}$.

The $C_I$ footprints observed in DNaseI protection experiments showed RNA polymerase bound primarily to the -35 hexamer and afforded little or no protection to
the -10 region of $P_{spolIG}$. Since RNA polymerase is believed to catalyze DNA melting at the -10 hexamer (Roe et al., 1985; Travers, 1987; Leirmo and Record, 1990) it is crucial that the polymerase contact this site to initiate transcription. In the absence of Spo0A(-P) this is largely prevented, probably because of the unusual length of the $P_{spolIG}$ spacer.

In general, point mutations within the spacer region do not effect promoter strength significantly (Siebenlist, et al., 1980b; Beutel and Record, 1990). However, it has been demonstrated that changes to the length of the spacer can have a strong influence on transcriptional activity (Stefano and Gralla, 1982; Ayers et al., 1989). Assuming a helical pitch of 10.5 bps per turn, the rotational positions of the -35 and -10 recognition elements are offset in a promoter with a normal 17 bp spacer region. Stefano and Gralla (1982b) studied the kinetics of transcription initiation with a series of promoter constructs that differed only in their spacer lengths. It was concluded that the rotational orientation of the recognition sites and the rigidity of the spacer DNA could be crucial determinants of promoter strength. They postulated that following closed complex formation, RNA polymerase maximizes -35 and -10 contacts by untwisting the spacer DNA and that the torque applied to the spacer creates stress within the DNA that could facilitate the nucleation of strand separation. The implications of this model were investigated by deHaseth and coworkers (Auble et al., 1986; Auble and deHaseth, 1988; Ayers et al., 1989) by testing the effects of structural changes to the spacer region on promoter activity. Their results are consistent with the notion that 17 bps is the optimal spacer length to permit polymerase binding to both recognition sites while providing enough torsion free energy to favor open complex formation. Stefano and Gralla speculated that promoters with sub-optimal spacer lengths might require activating proteins to adjust the helical pitch of the DNA to facilitate transcription initiation (Stefano and Gralla, 1982b).
Compared to the C₁ footprint, the DNaseI protection patterns of both C₁₁ and C₁₁Ⅲ indicated that the polymerase had extended its promoter contacts toward the -10 hexamer. The elongated footprint was accompanied by conspicuous sites of DNaseI hypersensitivity that were located within the spacer region. This suggested that Spo0A and Spo0A-P may have altered promoter structure or assisted the polymerase in distorting \( P_{spoIIG} \) so that it was able to contact the -35 and -10 recognition sites simultaneously.

A similar mode of action has been proposed for the MerR protein which regulates the transcription of a mercury resistance gene encoded by the transposon \( Tn501 \). Like the \( spoIIG \) operon, the \( mer \) gene is controlled by a promoter with an extended spacer (19 bps). Although RNA polymerase is able to bind the -35 hexamer of the \( mer \) promoter, stable contact with the -10 site is prevented by the long spacer (O'Halloran, et al., 1989; Frantz and O'Halloran, 1990). Chemical reagents that detect aberrations in DNA structure were used to demonstrate that when MerR binds to the spacer region in the presence of its allosteric effector, Hg(II), it contorts the DNA. The distortion has been interpreted as a localized unwinding of DNA which allows polymerase bound to the -35 hexamer to contact the -10 site. Experiments with mutant forms of MerR that are capable of unwinding \( P_{mer} \) DNA to varying degrees in the absence of Hg(II), have used to correlate increased DNA distortion with enhanced transcription. Therefore, it has been proposed that MerR activates the \( mer \) gene by removing a kinetic obstacle to transcription initiation through alignment of the -35 and -10 hexamers (Parkhill et al., 1993).

Since no DNaseI hypersensitive sites were evident in Spo0A(-P) (Figure 30) footprints it was apparent that, unlike MerR, neither form of the protein distorted \( P_{spoIIG} \) through DNA binding alone. Instead, the hypersensitive sites observed in C₁₁ and C₁₁Ⅲ footprints suggest that Spo0A(-P) may have assisted in untwisting the \( P_{spoIIG} \) spacer so that RNA polymerase bound to the -35 site was able to contact the -10 hexamer. Since
the effects of Spo0A and Spo0A-P on the completion levels and initiation kinetics at $P_{spoIIG}$ were substantially different, it was assumed that structural differences between C$_{II}$ and C$_{III}$ must be significant in terms of promoter activity. Given that the $P_{spoIIG}$ -35 sequence is situated on the opposite side of the helix compared to normal positioning relative the -10 element, the degree of spacer distortion required to align these sites would be considerable. This could account for the strong hypersensitive sites observed in the DNaseI protection pattern produced by the C$_{II}$ complex. However, most of the hypersensitive sites were absent in the C$_{III}$ footprint (Figure 36). Therefore, it is reasonable to assume that the degree of DNA distortion was reduced during the transition from C$_{II}$ to C$_{III}$. If torsional strain within C$_{II}$ was significantly higher than in C$_{III}$, it may account for the difference in complex stability that was apparent in the kinetic DNaseI footprinting assays conducted at 37°C. It may also suggest that non-phosphorylated Spo0A was unable to resolve strain within the C$_{II}$ ternary complex so that polymerase was prevented from initiating transcription efficiently.

The absence of DNaseI hypersensitivity from C$_{III}$ footprints and increased stability associated with the transition to C$_{III}$ suggested that resolution of the DNA distortion apparent in C$_{II}$ may be attributed to Spo0A-P. If the DNA in the $P_{spoIIG}$ spacer was underwound in order to align the -35 and -10 hexamers, one option to relieve structural tension in the template would be to induce melting of the DNA helix. However, because it was subject to heparin inhibition it does not appear that C$_{III}$ was equivalent to a "classical" open complex. From the studies of several E. coli promoters, open complexes are defined by exceptional stability and resistance to competitive inhibition. In many cases, open complex formation has been considered to be an irreversible step in the transcription initiation reaction (Buc and McClure, 1985; Straney and Crothers, 1987b). However, this is clearly not true for C$_{III}$ complexes formed at $P_{spoIIG}$.

A second possibility for resolving DNA distortion at $P_{spoIIG}$ would involve a release of polymerase contacts with the -35 hexamer. This would have the effect of relieving
the torsional strain caused by simultaneous contact with both promoter recognition sites and leave the polymerase free to catalyze strand separation at the -10 hexamer. Spo0A-P may compensate for release of the -35 hexamer by stabilizing the polymerase/complex through direct protein-protein contact. Freeing the polymerase from the -35 site may also contribute to rapid promoter clearance when the polymerase must release all contacts with promoter sequences as it converts from transcription initiation to RNA elongation. Whipple and Sonenshein (1992) proposed this to be the slow step in their generalized model for transcription initiation by B. subtilis polymerase. Upon close examination of the CIII footprint there appeared to be no indication of weakened DNaseI protection to the -35 hexamer (Figures 31, 32, & 36). However, it is very likely that the -40 region of a CIII complex would be inaccessible to DNaseI cleavage due to Spo0A-P binding to the OA box which overlaps the -35 sequence. It is possible that hydroxylradical footprinting which can produce high resolution protection patterns, may help to determine whether polymerase within a CIII complex has released or altered its contact with the -35 hexamer.

A model derived from the data of PspoIIIG transcription rate assays and DNaseI footprinting experiments is presented in Figure 43. It provides a working hypothesis for the influence of Spo0A(-P) on transcription based on the supposition that RNA polymerase in a closed complex is induced to untwist the spacer DNA (CII) and then resolve extensive template distortion (CIII) prior to the initiation of an RNA. Approximations of relative rates for various reaction steps in the presence or absence of Spo0A(-P) are given. Rates indicated for the formation of each of the ternary complexes reflects the rate of CI, CII, and CIII complex formation in kinetic footprinting experiments. Rates for the conversion of the various complexes to the initiated state were deduced from the rates of transcription initiation associated with the formation of these complexes.
This schematic depicts a model for transcription stimulation at $P_{spolIG}$ by the phosphorylated and nonphosphorylated forms of SpoOA. $R$ represents RNA polymerase and $P$ represents $P_{spolIG}$. Correlation between closed, untwisted, and resolved complexes with $C_I$, $C_{II}$, and $C_{III}$, respectively, represent interpretations of DNaseI footprinting involving SpoOA(-P) and RNA polymerase (Discussion III, 1). The velocities indicated for various reaction steps convey relative rates as determined from kinetic footprinting and transcription experiments. At present it is not known whether $C_I$, $C_{II}$, and $C_{III}$ are intermediates within a single reaction pathway or result from alternative pathways that depend on the presence and concentration of SpoOA(-P).
IV. The role of Spo0A-P phosphorylation.

1. N-terminal inhibition of the DNA binding domain.

Results from transcription assays showed that the level of $P_{spoII}G$ stimulation attributed to Spo0A$^{BD}$ was intermediate to the influence of Spo0A and Spo0A-P. Spo0A$^{BD}$ stimulated both completion levels and the rate of initiation, particularly at low KAc concentration (Results IV, 9 & 10). DNaseI protection studies have shown that binding of Spo0A$^{BD}$ to 0A boxes situated downstream of the abrB promoter produced the same footprint as the intact protein (Grimsley et al., 1994). Thus the transcription and footprinting data suggests that the C-terminal domain of Spo0A is capable of sequence-specific DNA binding and probably contains the portion of the protein that is crucial to transcription activation. Mutations that render Spo0A to be defective in transcription activation have been isolated in the C-terminus of the protein. One, called spo0A9V, is capable of repressing abrB expression in vivo but can not activate spoIIA transcription (Perego et al., 1991). It is also known that removal of the last 15 residues of the Spo0A protein causes a sporulation defective phenotype (Ferrari et al., 1985; Spiegelman et al., 1994). Thus, residues important for contact with RNA polymerase may reside in the C-terminus.

It is conceivable the N-terminal domain of Spo0A functions to negatively regulate transcription modulation by the C-terminal domain. This hypothesis was tested by placing a series of mutant spo0A genes that carried various deletions within the N-terminal domain, under the control of an inducible promoter. Under inducing conditions, some of these constructs allowed for the initiation of sporulation and the response was shown to be independent of a functional phosphorelay (Ireton et al., 1993). N-terminal deletions have also been implicated in the activation of various other transcription factors (Menon and Lee, 1990; Kahn and Ditta, 1991) suggesting that interactions between separate domains may be a common method of controlling the transcription regulating properties of these proteins.
A possible explanation for improved DNA binding and transcription stimulation by Spo0A-P is that phosphorylation modifies the structure of the protein thereby activating its transcription regulating properties. Although this has not yet been demonstrated, it has been proposed that the free energy change associated with aspartyl-phosphate bonds (-10 to -13 kcal/mol) is sufficient to drive a large conformational change in protein structure (Tanford, 1984; Stock et al., 1989). In its non-phosphorylated state the N-terminal domain of Spo0A may interfere with DNA binding by the C-terminus through steric hindrance or interact with residues in the C-terminal domain to hold it in an inactive conformation. Phosphorylation could induce an adjustment in the spatial orientation of the two domains leaving the C-terminus free to bind DNA and thus effect gene expression.

Despite the ability of Spo0A^BD to enhance the transcriptional activity of $P_{spoIIG}$, there were fundamental differences between the stimulatory effects of Spo0A^BD and Spo0A-P. In contrast to Spo0A-P, the ability of Spo0A^BD to stimulate the rate of initiation and completion level of transcription was extremely sensitive to KAc concentration. In addition, the kinetics of initiation in the presence of Spo0A^BD showed a distinct lag, particularly under low salt conditions, that was absent from rate assays containing Spo0A-P (Figure 28). The lag indicated that two reaction steps significantly influenced transcription initiation in assays that contained Spo0A^BD and that DNA binding and/or interaction with the polymerase was impaired. This would imply that despite its apparent inhibition of the function of the C-terminal domain, the N-terminal domain may also have an active role in the stimulation of $P_{spoIIG}$ transcription. It is conceivable that this portion of the protein may provide a stabilizing influence on Spo0A structure which may become critical at higher salt concentrations. DNase I protection experiments that examine the effects of Spo0A^BD on ternary complexes at $P_{spoIIG}$ at various KAc concentrations should provide clues to differences between the binding domain and Spo0A-P in terms of mediating transcription stimulation.
2. Possible roles for the 0A boxes upstream of $P_{\text{spoIIG}}$.

DNaseI footprinting experiments established that binding of Spo0A to $P_{\text{spoIIG}}$ particularly to the low affinity 0A boxes in region II, improved dramatically when the protein was phosphorylated. This finding is in agreement with the report of Baldus et al. (1994). DNA binding by various other response regulators including NtrC (NR1) and OmpR, has been shown to increase substantially following phosphorylation (Ninfa and Magasanik, 1986; Ninfa et al., 1987; Aibe et al., 1989). This raises the possibility that phosphorylation of Spo0A enhanced its capacity for transcription activation by increasing its affinity for specific 0A box(es) on the $P_{\text{spoIIG}}$ template.

Mutations within the 0A boxes situated at -94 and -84 had very little effect on the transcriptional activity of $P_{\text{spoIIG}}$ even though they abolished Spo0A(-P) binding to this region. In addition, DNaseI protection assays carried out with $P_{\text{spoIIG}}^{M94/84}$ template demonstrated that the 0A boxes at -94 and -84 were not essential for CII or CIII formation. Although it appeared that higher Spo0A(-P) concentrations were required to observe isomerizations in ternary structures on the mutated template. DNaseI assays suggested that the high affinity -94 and -84 0A boxes of region I may have influenced Spo0A-P binding to the low affinity 0A boxes of region II. It is conceivable that the binding sites in region I function as transient binding sites which localize the Spo0A protein to $P_{\text{spoIIG}}$. There has been speculation that multiple binding sites may effectively tether DNA binding proteins to their target sites. This could be particularly important when factors are present in a low intra-cellular concentration (Record and Mossing, 1987). A similar role has been postulated for NtrC enhancer sites in the activation of the $glnA$ promoter (Wedel et al., 1990). Alternatively, Spo0A-P bound to the high affinity sites may promote binding of additional Spo0A-P proteins to the 0A boxes in region II through cooperative interactions in a manner analogous to activation of the $P_{\text{RM}}$ promoter by the $\lambda$ repressor, cI (Discussion VII, 1.a.)
Transcription assays and DNaseI protection experiments identified the 0A boxes positioned at -50 and -40 as being the functionally important sites for stimulation of \( P_{spoIIG} \) transcription. However, it is difficult to determine from DNaseI protection patterns which sites must be occupied by Spo0A(-P) to catalyze C\(_{II}\) or C\(_{III}\) formation because the -60 to -20 region was protected from DNaseI cleavage by RNA polymerase. Because Spo0A-P appeared to bind equally well to the -50 or -40 0A boxes in the absence of RNA polymerase, it is reasonable to assume that Spo0A-P binds both sites to facilitate C\(_{III}\) formation. Although non-phosphorylated Spo0A bound poorly to the low affinity sites in the absence of RNA polymerase it is probable that the protein must bind at least one site to convert C\(_{I}\) into a C\(_{II}\) complex.

It is possible that the 0A boxes at -50 and -40 contribute separate functions (i.e., promoter distortion versus the resolution of DNA distortion) to the isomerization of ternary complexes formed at \( P_{spoIIG} \). Spo0A(-P) may be required to bind one site to induce the polymerase to untwist the \( P_{spoIIG} \) spacer leading to C\(_{II}\) formation. Binding to a second 0A box may be necessary to resolve DNA distortion perhaps through direct interaction with the polymerase, thus leading to C\(_{III}\) formation. Non-phosphorylated Spo0A may have been unable to accommodate the isomerization to C\(_{III}\) because it could only bind one of the functional 0A boxes. This hypothesis is consistent with the observation that transitions from C\(_{I}\) to C\(_{II}\) or C\(_{III}\) were dependent on Spo0A-(P) concentration. Very low concentrations of Spo0A-P produced a mixture of C\(_{II}\) and C\(_{III}\) footprints which could have resulted if the amount of Spo0A-P was insufficient to bind both of the 0A boxes in all ternary complexes formed in the reaction. Interestingly, DNaseI hypersensitivity that distinguished the C\(_{II}\) complex appeared to be reduced at very high concentrations of nonphosphorylated Spo0A suggesting that weak binding to a site crucial for the C\(_{II}\) to C\(_{III}\) isomerization was overcome under this condition. It should be possible to test the above hypothesis by examining transcription from \( P_{spoIIG} \) constructs with mutations in the -50 and -40 0A boxes. However, mutating the -40 site
has the caveat that the -35 polymerase recognition site would be altered thus making it difficult to access changes to $P_{spoIIG}$ activity.

3. Evidence for protein-protein contact.

Even if the two 0A boxes of region II contribute separate functions required for isomerization to $C_{\text{III}}$ and hence stimulation of $P_{spoIIG}$ transcription, it is possible that Spo0A-P stimulates promoter activity because it is able to assist the polymerase through specific protein-protein contacts. Spo0A may be incapable of providing a similar function. Kinetic footprinting experiments demonstrated that very little Spo0A bound to $P_{spoIIG}$ in the absence of RNA polymerase (Figures 31 & 32). Once polymerase was added to the reaction, binding of Spo0A to the 0A boxes at -94 and -84 was enhanced and a relatively slow transition to a $C_{\Pi}$ DNaseI protection pattern followed. This transition was interpreted as evidence that Spo0A bound to at least one of the 0A boxes in region II, and indicates that stable binding of Spo0A to this site resulted from synergistic interaction with the polymerase.

The interaction between Spo0A and the polymerase is similar to that reported for the catabolite activator protein (CAP) and RNA polymerase at the lac promoter of E. coli. In vitro studies have demonstrated that binding of CAP alone to the lac promoter was subject to a very high rate of dissociation (Straney et al., 1989). Addition of RNA polymerase to binding reactions stabilized binding of CAP to the DNA and led to the production of transcriptionally competent ternary complexes. It was proposed that synergy, resulting from direct protein-protein contact, stabilized binding of both CAP and polymerase to the DNA. It was determined that CAP then catalyzed a rapid isomerization to an open complex.

In the case of Spo0A(-P), there was no evidence that binding to $P_{spoIIG}$ resulted in DNA distortion in the absence of RNA polymerase (Figure 30). Instead, the strong -28 and -26 DNaseI hypersensitive sites of $C_{\Pi}$ and $C_{\text{III}}$ footprints which are 10 to 12 bps downstream from the nearest 0A box (at -40), suggested that Spo0A(-P) induced RNA
polymerase to distort $P_{\text{spolIG}}$ spacer DNA. Given the proximity of the downstream 0A boxes to the region of RNA polymerase binding, it is simplest to assume that Spo0A(-P) directed a conformational change in the polymerase through direct protein-protein contacts and that this resulted in DNA deformation.

Evidence for protein-protein contact between RNA polymerase and several different bacterial transcription factors has been reviewed recently by Ishihama (1993). Mutational analysis of RNA polymerase has shown that transcription factors often fall into one of two classes. Class I factors such as OmpR and OxyR, contact the C-terminus of an $\alpha$ subunit within the polymerase to affect transcription initiation (Igarashi, et al., 1991; Ishihama, 1993). Whereas class II factors including PhoB and $\lambda$CI, interact with the $\sigma$ subunit (Makino et al., 1993; Li et al., 1994). There is genetic evidence that hints at interaction between $\sigma^A$ and at least some spo0 gene products (Kawamura et al., 1985). However, there are still a number of transcription factors including MerR and $\lambda$CII which have not yet been characterized (Ishihama, 1993). These factors may exhibit new ways of contacting RNA polymerase and Spo0A-P may prove to belong in this category. Protein subunit crosslinking studies have been undertaken in our laboratory and may determine which portion of the polymerase is touched by Spo0A(-P).

V. Temperature and salt effects on ternary complex formation and transcription from $P_{\text{spolIG}}$

1. Temperature effects on ternary complex formation.

DNaseI protection assays carried out at 31° and 25° identified the Spo0A dependent transition from $C_I$ to $C_{II}$ as a temperature sensitive step. In contrast, the isomerization to $C_{III}$ facilitated by Spo0A-P was unaffected at these temperatures. These observations appear to be inconsistent with in vitro transcription data which indicated that promoter activity was dramatically reduced at temperatures below 30° C even in the presence of Spo0A-P. The reason for this discrepancy is not known. It is possible that the transition
from \( C_{II} \) or \( C_{III} \) complexes to the heparin-resistant state which must occur sometime after incorporation of the first GTP, may be very sensitive to reduced temperature. Since \( C_{III} \) is not perceived to be a stable open complex, the transition from \( C_{III} \) to HR probably involves opening the DNA helix which is known to be an endothermic process (Leirmo and Record, 1990). Because of the extended \( P_{spoIIIG} \) spacer, the enthalpic cost of opening the helix may be higher than usual.

The 31° and 25° C DNaseI experiments produced a second paradox when it was noted that the \( C_I \) and \( C_{II} \) footprints did not disappear late in the time-course as they had at 37° C. This implied that lower temperatures stabilized \( C_I \) and \( C_{II} \) complexes even though the rate and level of \( C_{II} \) formation was inhibited. There is no obvious explanation for complex stabilization unless the rate of decay in RNA polymerase activity was significantly reduced at the lower temperatures.

DNaseI protection experiments with RNA polymerase, carried out at 15° C, produced \( C_I \) footprints that were comparable to those obtained at higher temperatures. In contrast, the Spo0A(-P) dependent formation of \( C_{II} \) or \( C_{III} \) type footprints was completely inhibited at 15° C. At this temperature, DNaseI protection patterns suggested that RNA polymerase and Spo0A(-P) were able to bind \( P_{spoIIIG} \) but the ternary complexes could not isomerize to \( C_{II} \) or \( C_{III} \). It was concluded that low temperature adversely affected Spo0A(-P) mediated isomerizations involving changes in the polymerase conformation and/or DNA structure.

Footprinting techniques have been used previously to study the effects of low temperature on ternary complexes formed at various \( E. coli \) promoters. Three distinctly different footprint patterns were obtained at the A1 promoter from phage T7 at various temperatures (Schickor et al., 1990). The dissimilar footprints were postulated to arise from closed (<8° C), intermediate (8° - 21° C), and open complexes (>21° C). Similarly, it was found that RNA polymerase produced different footprints at the \( lacUV5 \) and T7-A3 promoters at 0° versus 37° C (Kovacic, 1987). In each case, the
polymerase footprints obtained at the lowest temperatures protected a smaller region of the promoter from chemical or nuclease cleavage than at the higher temperatures. Interestingly the low temperature footprints were primarily confined to the -35 region and thus resembles the C_I footprint observed at P_{spoII}. Higher temperatures facilitated an extension of the polymerase footprint to the -10 region and the start-site for transcription. The effect was similar to the influence of Spo0A(-P) on polymerase complexes formed at P_{spoII} (>25° C) which produced the extended C_{II} and C_{III} footprints.

These similarities indicate that the abnormal structure of P_{spoII} imposes a considerable thermodynamic barrier to transcription initiation. The extended spacer which must be untwisted over a very short distance, may exact a high enthalpic cost. If so, most RNA polymerase/P_{spoII} associations may remain trapped as closed complexes because they are unable to attain the activation energy necessary for isomerization. Spo0A and Spo0A-P could function as catalysts to lower the activation energy requirement to overcome the unfavorable change in enthalpy associated with isomerizations in protein or DNA structure. This possibility is consistent with interpretations of transcription rates assays discussed above (Discussion II, 2).

2 The effect of ion concentration.

Thermodynamic studies of three different E. coli promoters have demonstrated that the enthalpy of transcription initiation is positive. Consequently, transcription initiation is an entropically driven process. This accounts for the strong salt dependencies reported for most in vitro promoter studies (Roe et al., 1985; Buc and McClure 1985; Duval-Valentin and Ehrlich 1987). In general, the salt sensitivity of protein/DNA binding interactions are dominated by the mixing entropy associated with cation release resulting in the polyelectrolyte effect discussed earlier (Results III, 2). This is likely to be the basis for the extreme sensitivity of P_{spoII} transcription to elevated concentrations of NaCl and KCl. However, the finding that salt sensitivity could be
relieved by the substituting Ac\(^-\) for Cl\(^-\) showed that anions could also have a strong
influence on the initiation process at \(P_{\text{spoIIIG}}\). Because this effect was observed only in
the presence of Spo0A-P it appeared that the Ac\(^-\) effect exposed a fundamental
difference in the mechanism of transcription stimulation by Spo0A-P as compared to
Spo0A or Spo0ABD.

Substitution of glutamate (Glu\(^-\)) or Ac\(^-\) for Cl\(^-\) has been shown to stabilize DNA
binding by RNA polymerase, the lac repressor, and various restriction enzymes (Leirmo
\textit{et al.}, 1987; Leirmo and Record, 1990; Ha \textit{et al.}, 1992). In addition, anion substitution
often expands the range of salt concentrations over which the enzymatic activities of
DNA binding proteins can occur. Since significant DNA/anion interactions are unlikely
to occur, the anion effect is believed to involve DNA/protein interactions. The influence
of anion type on protein/DNA binding usually follows the Hofmeister series which ranks
anions according to their effects on protein solubility (Leirmo and Record, 1990).
Record and coworkers have investigated the thermodynamic origins of the anion effect
by studying the influence of anion substitution on DNA binding by the lac repressor (Ha
\textit{et al.}, 1992). They determined that there was no straightforward explanation for the
anion effect. However, one hypothesis raises intriguing possibilities for the KAc effect
on transcription stimulation at \(P_{\text{spoIIIG}}\).

It has been speculated that various types of anions (particularly Glu\(^-\) and Ac\(^-\)) may be
preferentially excluded from non-polar amino acid residues that are exposed to the
solvent. This would produce a gradient in water concentration in close proximity to the
surface of the protein. The magnitude of the gradient would increase with elevated Ac\(^-\)
concentrations and could provide a contribution to the thermodynamics of DNA binding
interactions and/or conformational changes in protein structure if either results in the
burial of hydrophobic surface area. The free energy change associated with "the
hydrophobic effect" would depend on the type of anion in solution and the size of the
hydrophobic surface area removed from solvent contact (Leirmo and Record, 1990; Ha et al., 1992).

If the hydrophobic surface area of Spo0A-P was significantly greater than either Spo0A or Spo0A^{BD} it follows that the hydrophobic effect would exert a larger influence on its activities. Structural modeling of Spo0A by P. Youngman and coworkers led them to speculate that phosphorylation could expose a portion of the Spo0A protein that contains several hydrophobic residues (Green et al., 1991). If so, it is possible that phosphorylation of Spo0A could be responsible for driving a protein conformational change that would provide for a strong hydrophobic effect. This could translate into increased affinity for the functional 0A boxes (region II) of *P_{spoII}G* that would be less sensitive to Ac^{-} concentration. Alternatively, a hydrophobic effect could influence a conformational change in Spo0A-P after it had bound to target sites on the DNA. A structural change in Spo0A-P could be coupled to DNA distortion or a conformational change in the polymerase which in turn would lead to C_{III} formation and stimulation of *P_{spoII}G* transcription.

Since all DNaseI protection experiments described in this work were carried out at low concentrations of KAc it is not known whether high salt concentrations inhibited DNA binding by non-phosphorylated Spo0A, Spo0A^{BD} and/or RNA polymerase. It is also possible that higher KAc concentrations might effect the synergistic interaction that was apparent between Spo0A and the polymerase and which led to C_{II} formation. It should be informative to carry out DNaseI protection experiments at increased KAc concentrations to determine whether protein binding or the isomerization from C_{I} to C_{II} is effected.
VI. The effect of Spo0A-P on $P_{spoIIA}$.

1. Stimulation by Spo0A-P and the effect of $\sigma^H$ concentration.

*In vitro* transcription assays demonstrated that Spo0A-P stimulated the transcriptional activity of $P_{spoIIA}$. Although the mechanism of stimulation was not investigated it was apparent that the difference between basal levels of transcription and the effect of Spo0A-P was dependent on $\sigma^H$ concentration. Transcription assays that contained various amounts of $\sigma^H$ showed that the stimulatory effect of Spo0A was greatest at low $\sigma^H$ concentrations. Transcription assays that contained very high $\sigma^H$ concentrations resulted in $P_{spoIIA}$ activity that approached levels obtained from assays that contained Spo0A-P. This result may reflect technical aspects of the *in vitro* transcription system but could also have relevance to the way expression of the *spoIIA* operon is regulated *in vivo*.

There is a potential for Spo0A-P to positively regulate the *spoIIA* operon directly through stimulation of transcription initiation at $P_{spoIIA}$, and indirectly by influencing expression of *spo0II* (the $\sigma^H$ gene). During vegetative growth, *spo0II* expression is repressed by AbrB which ensures that *spoIIA* and the remainder of the $\sigma^H$ regulon is silent. Because Spo0A is a negative regulator of *abrB* during the transition state it can assist the induction of *spoIIA* by facilitating the production of $\sigma^H$. Thus, the effect of Spo0A-P at $P_{spoIIA}$ may simply augment *spoIIA* expression until the intracellular concentration of $\sigma^H$ is sufficient to direct transcription of the operon.

VII. Spo0A-P as a model for transcription factor activity.

1. Review of prokaryotic transcription activator proteins.

To compare activities of Spo0A to the function of transcription factors in general, it will be useful to review what is known about some of the more well characterized transcriptional activators.
1.a. The λ cI protein.

The λ repressor, or cI protein, is both an activator and repressor of transcription. cI binds cooperatively to its operator sites OR1 and OR2 that are superimposed over divergent promoters. In this way, cI represses PR, a promoter for genes crucial to the lytic cycle, and activates its own synthesis through positive regulation of PRM. Hawley and McClure examined the kinetics of transcription initiation at PRM and concluded that cI did not affect binding of RNA polymerase but did stimulate the conversion of closed to open complexes (Hawley and McClure, 1982). cI was not required to stabilize open complexes once they were formed. However, it was observed that binding of cI to the OR2 site that overlaps the -35 sequence of the promoter, was stabilized by open complexes at PRM. This was interpreted as evidence for protein-protein contact between the polymerase and cI.

Further evidence for contact between the polymerase and cI came from mutational analyses of the activator protein. Several mutant forms of cI were isolated that were not impaired in their ability to bind OR sites or repress transcription from PR, but were deficient in activating PRM (Guarente et al., 1982; Hawley and McClure, 1983b; Hochschild et al., 1983; Bushman et al., 1989). These alleles were found to contain point mutations which clustered near a 'helix-turn-helix' DNA binding domain indicating that this portion of the protein (the activator region) interacted with RNA polymerase to affect transcription at PRM. Recently, Susskind and colleagues reported the isolation of a mutant form of RNA polymerase that restored gene activating properties to a cI a protein with a mutated activator region (Li et al., 1994). The suppressor mutation was found to be in the C-terminus of the σ70 subunit near the helix-turn-helix domain that is believed to bind the -35 promoter sequence. Thus, it appears that cI bound to OR2 contacts the σ70 subunit of RNA polymerase to catalyze the isomerization from a closed to an open complex.
1.b. The catabolite gene activator protein (CAP or CRP).

Despite being one of the most intensively studied transcription factors, the mechanism through which the catabolite gene activating protein (CAP or CRP) regulates gene expression in response to carbon utilization is still a matter for conjecture (Reznikoff, 1992). A survey of genes within the CAP regulon has demonstrated that the positioning of CAP binding sites positioned near activated promoters varies from -70 to -40 (Collado-Vides et al., 1991). CAP binds to DNA in the form of a dimer which is dependent on the allosteric effector, cyclic AMP (cAMP). It has been reported that the equilibrium constant for site specific binding of CAP to the lac promoter region increases by two orders of magnitude over what is considered to be a physiological range of cAMP concentrations (0.5 - 10 μM) (Fried and Crothers, 1984). However, investigations into the kinetics of transcription initiation at the major lac promoter (lacP1) by different assay techniques has produced contradictory results regarding which reaction step is stimulated by CAP (Malan et al., 1984; Straney et al., 1989).

The crystal structure of a CAP-DNA complex has been solved (Schultz et al., 1991) and it suggests that CAP causes a 90° bend in the DNA. There has been speculation that DNA bending is an integral part of the CAP activation mechanism and either orients the protein with respect to the polymerase to optimize protein-protein contact or allows the polymerase to contact DNA sites upstream of the CAP binding site (Gartenberg and Crothers, 1991; Schultz et al., 1991). There is general agreement that binding of RNA polymerase to the lac promoter region stabilizes site specific DNA binding by CAP which has been interpreted as evidence for direct protein-protein contact. (Ren et al., 1988; Straney et al., 1989). As for cI, CAP mutants have been isolated which bind DNA normally but are defective in activating transcription (Irwin and Ptashne, 1987; Eschenlauer and Reznikoff, 1991). These mutations were found to lie near the DNA binding domain. The possibility of contact between CAP and the polymerase has been examined by studying cooperative binding between CAP and a mutant form of RNA
polymerase. Interestingly, CAP and the mutant polymerase which contained a truncated \( \alpha \) subunit, bound cooperatively to the \( galP1 \) promoter but not to \( lacP1 \) (Kolb \textit{et al.}, 1993) indicating that CAP has the potential to affect transcription initiation by two different mechanisms. One involving direct contact with \( \alpha \) subunit of the polymerase and the other through an undefined mechanism.

1.c. The OmpR activator.

OmpR is a response regulator which controls the reciprocal regulation of the outer membrane porins, \( ompC \) and \( ompF \), according to changes in medium osmolarity (Stock \textit{et al.}, 1989). At low osmolarity OmpR stimulates transcription of \( ompF \). At high osmolarity OmpR represses \( ompF \) and activates \( ompC \) transcription. The activities of OmpR are controlled by the sensor/kinase, EnvZ, which has both kinase and phosphatase activity. The current view is that the balance between the kinase and phosphotase activities of EnvZ is affected by osmolarity (Russo and Silhavy, 1991; Russo and Silhavy, 1993). Consequently, EnvZ is able to maintain, raise, or lower the intracellular concentration of OmpR-P depending on the conditions of the external environment. Phosphorylation of OmpR has been shown to enhance its ability to bind DNA and it appears that the protein functions to control relative levels of \( ompF \) and \( ompC \) transcription from low and high affinity binding sites upstream of their respective promoters (Aiba \textit{et al.}, 1989; Nakashima \textit{et al.}, 1991).

The -35 and -10 promoter recognition sites of the \( ompF \) and \( ompC \) promoters deviate considerably from consensus promoter sequences. Therefore, the mechanism through which OmpR-P activates transcription was investigated by fusing its DNA binding site to 27 synthetic promoter sequences (Tsung \textit{et al.}, 1990). OmpR was observed to activate \textit{in vivo} transcription from most promoter constructs although one which contained a consensus -35 hexamer was actually repressed by OmpR. The conclusion from \textit{in vivo} footprinting experiments was that OmpR stimulated transcription by stabilizing closed complex formation through protein-protein contact. Evidence for
direct contact between OmpR and RNA polymerase has also resulted from a mutational analysis of the polymerase. OmpR was unable to assist RNA polymerase that contains mutations within the C-terminus of the α subunit in transcribing the _ompF_ promoter (Slauch _et al._, 1991). This indicated that, like CAP, OmpR functions to activate transcription through direct contact with the α subunit of the polymerase.

1.d. The NtrC activator.

NtrC (NR1) of enteric bacteria, activates genes necessary to nitrogen assimilation and is probably the most extensively investigated of all response regulators. Its transcription regulating activities are controlled by the sensor/kinase, NtrB (NRII), which senses nitrogen limitation (Ninfa and Magasanik, 1986). Investigations into gene activation by NtrC have concentrated on the _glnA_ gene (glutamine synthetase). Activation of _glnA_ has been shown to depend on two NtrC binding sites located 140 and 110 upstream of the transcription start site. These binding sites have been demonstrated to have enhancer-like properties and can be moved a kilobase upstream or downstream from their normal positions without eliminating NtrC mediated activation of _glnA_ expression (Reitzer and Magasanik, 1986; Ninfa _et al._, 1987). Kustu and colleagues have demonstrated that NtrC bound to the enhancer sites contacts RNA polymerase at the _glnA_ promoter through DNA looping (Wedel _et al._, 1990; Weiss _et al._, 1992b).

NtrC interacts with a form of RNA polymerase that contains a sigma subunit called σ^{54}. σ^{54} is unique among sigma subunits because it is incapable of denaturing promoter DNA without the assistance of an activator protein (Helman and Chamberlin, 1988; Kustu _et al._, 1989). It has been demonstrated that NtrC couples the hydrolysis of ATP to the conversion of closed to open Eσ^{54}/promoter complexes (Kustu _et al._, 1991; Weiss _et al._, 1991; Austin and Dixon, 1992). The ATPase activity of NtrC was found to be dependent on phosphorylation and DNA binding. It has been postulated that phosphorylation causes NtrC subunits to oligomerize which facilitates cooperative DNA binding interactions (Porter _et al._, 1993; Porter _et al._, 1994). Mutational analysis has
identified residues within the central domain of NtrC as being important to oligomerization. Removal of the N-terminal domain which contains the site of phosphorylation, renders the protein inactive suggesting that it plays an active role in transcription activation. DNA binding is mediated through a helix-turn-helix domain in the C-terminus of the protein.

2. Spo0A-P may stimulation transcription initiation two different ways.

Spo0A appears to be the first transcription factor known to activate gene expression by interacting with different forms of RNA polymerase holoenzyme. This unique property may require that it utilize two different mechanisms to influence transcription initiation. In this regard, Spo0A may be similar to CAP which also appears to affect transcription initiation through two mechanisms (Discussion VII, 1b.). If Spo0A is shown to directly contact the $\sigma^A$ subunit of RNA polymerase bound to $P_{spoII}$, it seems unlikely that it would be able to interact with the $\sigma^H$ subunit in the same way unless contact is made in a region that is highly conserved in both sigma factors. A survey that compared all $\sigma^A$ and $\sigma^H$-type promoters known to depend on Spo0A for activation, revealed that their putative 0A boxes are usually oriented in opposite directions (Spiegelman et al., 1994). This could indicate that different Spo0A residues are required for protein-protein contacts with either the $\sigma^A$ or $\sigma^H$ forms of RNA polymerase.

The discovery that Spo0A-P catalyzes the conversion ternary complexes at $P_{spoII}$ indicates that it is functionally more similar to CI rather than OmpR, which has been shown to affect polymerase binding (Discussion VII, 1.a. & 1.c.). Like CI, Spo0A-P appears to exhibit cooperative binding to multiple binding sites near its target and may contact the sigma subunit of the polymerase to enhance transcription initiation. However, Spo0A depends on phosphorylation to enhance its DNA binding capability. DNaseI footprinting suggested that phosphorylation may enable Spo0A-P that is bound to the high affinity 0A boxes of region I, to facilitate binding of additional subunits to the low affinity sites of region II (Figure 37). It is possible that phosphorylation may
affect cooperative interaction between monomeric forms of Spo0A in a manner analogous to the oligomerization of NtrC (Discussion VII, 1.d.). Gel filtration experiments carried out with Spo0A and Spo0A-P have determined that there is no evidence that phosphorylation causes Spo0A to oligomerize while in solution (Grimsley et al., 1994). However, this does not exclude the possibility that Spo0A-P is able to oligomerize on the DNA.

It is not likely that Spo0A-P has ATPase activity since it lacks an ATP binding motif. This property may be limited to transcription factors like NtrC that activate σ^54 promoters. It appears that Spo0A-P is not required to catalyze the formation of a stable open complex but rather increases the concentration of an intermediate complex which is capable of initiating transcription rapidly. Given the constraints imposed by the extended spacer of P_{spoIIG}, it is possible that Spo0A-P functions in an analogous manner to MerR (Discussion III, 1) and stimulates transcription initiation by assisting the polymerase to untwist or otherwise distort the promoter DNA.

Perhaps the most intriguing outcome of investigations into the activities of different transcription factors is that rather than revealing similarities, activating systems are more remarkable for their diversity. This diversity likely reflects differences in the intrinsic properties of transcription factors, sigma factors, and the promoters they activate. The transcription initiation process may be best viewed as a series of reaction steps which provides multiple points at which to exert a regulatory influence. Consequently, activating proteins can stimulate the process by influencing the binding of the original reactants or by interacting with a specific reaction intermediate to drive the reaction toward productive initiation.
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Appendix

I. Reaction kinetics.

1. The kinetics of transcription initiation.

The purpose of this appendix is to show the mathematical treatment of the data from experiments measuring heparin-resistant complexes at \( P_{spoIIG} \). This procedure was used to measure the overall rate of heparin-resistant complex formation and provided for an investigation of the effects of Spo0A(-P) on the kinetics of transcription initiation at \( P_{spoIIG} \) (tau analysis). The following derivations have been published previously and provide a method of kinetic analysis that has been widely used to study transcription initiation (McClure, 1980; Hawley and McClure, 1980; Stefano and Gralla, 1980). This review closely follows the mathematical treatment of transcription initiation kinetics as described by Wellington (1991).

2. Review of the kinetics of first-order reactions.

A first order decay reaction may be represented by;

\[
\begin{align*}
A & \xrightarrow{k} B \\
\end{align*}
\]  

(A-1)

where the rate of the reaction would be described by the equation;

\[
\frac{d[A]}{dt} = -k[A].
\]  

(A-2)

Rearrangement of (A2) followed by integration of both sides allows for \([A]\) (or \([B]\)) to be determined at any \( t \) (\([A] = [A]_0 \) at \( t = 0 \));

\[
\int_{[A]_0}^{[A]} \frac{d[A]}{[A]} = -k \int_0^t dt.
\]  

(A-3)
This equation is solved by:

\[ \ln[A] - \ln[A]_0 = -kt \quad (A-4) \]

or the equivalent expression:

\[ [A] = [A]_0e^{kt} \quad (A-5) \]


The overall process of heparin-resistant complex formation at \( P_{spoIIIC} \) may be reduced to a simple isomerization reaction

\[ HS \xrightleftharpoons[k_r]{k_f} HR \quad (A-6) \]

As noted previously, this is valid only if all the steps in the overall reaction are either true or pseudo first-order and that one step in A-6 equilibrates slowly relative to the others (Results IV, 2). Under these conditions;

\[ \frac{d[HR]}{dt} = k_f[HS] - k_r[HR] \quad (A-7) \]

and;

\[ [P_T] = [HS] + [HR] \quad (A-8) \]
where $PT$ is the total concentration of promoter template, $HS$ is a heparin-sensitive complex, and $HR$ is a heparin-resistant complex.

Rearrangement of the expression (A-8) followed by substitution into (A-7) gives:

$$\frac{d[HR]}{dt} = - (k_f + k_r)[HR] + k_f[PT]. \quad (A-9)$$

This equation has the form

$$\frac{d[HR]}{dt} = - \left(\frac{1}{\tau}\right)[HR] + \beta \quad (A-10)$$

which has the same form as $d[A]/dt = -k[A]$ (Appendix I, 2) and is solved by the equation;

$$\ln([HR] - \beta \tau) - \ln([HR]_0 - \beta \tau) = - \left(\frac{1}{\tau}\right)t \quad (A-11)$$

or the equivalent expression;

$$\ln\left(\frac{[HR] - \tau \beta}{[HR]_0 - \tau \beta}\right) = - \left(\frac{1}{\tau}\right)t. \quad (A-12)$$

In considering the reaction model (A-6) it can be seen that after infinite reaction time $d[HR]/dt = 0$ and $\beta \tau$ will be equal to the final concentration of heparin-resistant complexes at reaction completion ([HR]$_\infty$). Given that the initial concentration of heparin-resistant complexes was zero, substitution of $\beta \tau$ and [HR]$_0$ and rearrangement gives;

$$\ln\left(1 - \frac{[HR]}{[HR]_\infty}\right) = - \left(\frac{1}{\tau}\right)t. \quad (A-13)$$
This expression describes the rate of approach to reaction completion for transcription rate assays. A plot of $\ln(1 - [HR]/[HR]_\infty)$ versus $t$ should produce a straight line equal to the value for $-(1/\tau)$ (or $-k_{obs}$), the overall rate constant for the production of heparin-resistant complexes.

4. Interpretation of $1/\tau$.

As noted previously, it was assumed that the formation of a heparin-resistant complex was preceded by a heparin-sensitive complex which resulted from RNA polymerase binding to $P_{spoIIIG}$ (Results IV, 2.). Under conditions of RNA polymerase excess, the two step reaction model for heparin-resistant complex formation (Results IV, 3.) becomes:

$$
P \overset{[R]k_1}{\underset{k_{-1}}{\rightleftharpoons}} HS \overset{k_f}{\underset{k_r}{\rightarrow}} HR$$

because the concentration of free polymerase in the assay does not change appreciably over the duration of the reaction ($[RT] = \text{constant}$). Assuming that $[P] + [HS] + [HR] = [P_T]$, then;

$$\frac{d[P]}{dt} = k_{-1}[HS] - k_1[R][P]$$

$$\frac{d[HS]}{dt} = k_1[R][P] - (k_{-1} + k_f)[HS] + k_r[HR]$$

$$\frac{d[HR]}{dt} = k_f[HS] - k_r[HR]$$

Applying the steady state assumption to equation (A16) (the change in [HS] is negligible) followed by rearrangement gives;
\[(k_{-1} + k_f) [HS] = k_1[R][P] + k_r[HR]. \quad (A18)\]

Since \([P] = [P_T - HS - HR]\), substitution of this expression into (A18) followed by rearrangement gives;

\[[HS] = \frac{k_1[R][P_T] + (k_r - k_1[R])[HR]}{k_1[R] + k_{-1} + k_f}. \quad (A19)\]

When the expression for \([HS]\) above is substituted into equation (A17) it yields;

\[
\frac{d[HR]}{dt} = - \left( \frac{k_1[R](k_f + k_r) + k_{-1} k_r}{k_1[R] + k_{-1} + k_f} \right)[HR] + \frac{k_f k_1[R][P_T]}{k_1[R] + k_{-1} + k_f}. \quad (A20)
\]

This expression has the general form \(d[HR]/dt = -(1/\tau) [HR] + \beta\). As discussed above, this differential equation is solved by \(\ln (1 - [HR]/[HR]_\infty) = -(1/\tau)t\). Therefore, \(1/\tau\) describes the rate of approach to reaction completion. From equation (A20) it is apparent that;

\[
\frac{1}{\tau} = \frac{k_1[R](k_f + k_r) + k_{-1} k_r}{k_1[R] + k_{-1} + k_f}. \quad (A21)
\]

However, if it is assumed that \(k_f \gg k_r\) and that the product \(k_{-1}k_r\) is extremely small compared to \(k_fk_1[R]\), this expression is reduced to;

\[
1/\tau = \frac{k_1[R] k_f}{k_1[R] + k_{-1} + k_f} \quad (A22)
\]

or its reciprocal form;
\[
\tau = \frac{k_{-1} + k_\ell}{k_\ell k_1} \left( \frac{1}{[R]} \right) + \frac{1}{k_\ell}
\] (A23)

Therefore, a plot of \( \tau \) versus \( 1/[R] \) should produce a straight line with an ordinate intercept equal to \( 1/k_\ell \).