SPO0A REPRESSION OF TRANSCRIPTION FROM THE *BACILLUS SUBTILIS ABRB* PROMOTER IS MEDIATED BY SPO0A TETHERING RNA POLYMERASE TO THE PROMOTER REGION

by

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1. Abstract

Bacillus subtilis forms an endospore as a response to nutritional stress. Spore formation produces only one progeny cell, therefore it is advantageous for cells to avoid sporulating if other mechanisms for survival are available. The *abrB* gene product is involved in repressing sporulation. Transcription of *abrB* is repressed by the Spo0A protein. Spo0A is present in the cell at low levels during vegetative growth, and is activated by phosphorylation. The purpose of this thesis was to define the mechanism by which phosphorylated Spo0A represses *abrB* transcription.

There are two Spo0A concensus binding sites (OA boxes) downstream from the *abrB* promoter and a third OA box upstream from the promoter. Based on this structural information, three models for Spo0A(~P) mediated inhibition of *abrB* transcription were developed. In the first model a DNA loop is formed by protein:protein interactions between Spo0A(~P) bound at the upstream and downstream OA boxes, blocking RNA polymerase binding at the *abrB* promoter. The second model proposed that Spo0A(~P) binds the downstream OA boxes, blocking RNA polymerase from binding to the *abrB* promoter region. In the third model RNA polymerase and Spo0A(~P) bind the *abrB* promoter region simultaneously. Spo0A(~P) prevents transcription from proceeding by "tethering" polymerase at the promoter.

The DNA looping model was tested by measuring Spo0A(~P) repression of *abrB* transcription on promoter fragments with or without the upstream OA box. Transcription of *abrB* was inhibited in the absence of the upstream OA box, therefore DNA looping between the upstream and downstream OA boxes was not necessary for repression of *abrB* transcription. To determine whether RNA polymerase and Spo0A(~P) bound to the *abrB* promoter simultaneously, the ability of the two proteins to protect *abrB* DNA from cleavage by DNaseI or

hydroxyl radical was explored. If binding of RNA polymerase and Spo0A(~P) were found to be mutually exclusive then the blocking model would be the most accurate, however, both proteins protected *abrB* promoter regions simultaneously therefore RNA polymerase was shown to be tethered at the *abrB* promoter by Spo0A(~P).

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COMMON ABBREVIATIONS

bp=base pairs

BSA=bovine serum albumin

EDTA=ethylenediaminetetraacetic acid pH 8.0

EtBr=ethidium bromide

KAc=potassium acetate

MgAc=magnesium acetate

PAGE=polyacrylamide gel electrophoresis

SDS=sodium dodecyl sulfate

Spo0A(~P)=Spo0A or Spo0A~P

TBE=0.09 M Tris base, 0.002 M EDTA, 0.09 M borate pH 8.0 (Sambrook et al, 1989)

TE=10 mM Tris pH 8.0, 1mM EDTA pH 8.0 (Sambrook et al, 1989)

Tris=tris(hydroxymethyl)aminoethane

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1. Introduction

1.1. Sporulation in *Bacillus subtilis*

Bacillus subtilis is an organism which forms an endospore as a response to nutritional stress. Spore formation is the result of a modified asymmetric cell division, such that one daughter cell differentiates into the spore while the other contributes to spore formation and is then sacrificed (Errington, 1993). The sporulation process takes a number of hours and produces only one progeny cell rather than the two that would be produced from a normal cell division. Sporulation is an inefficient mechanism for bacterial growth, therefore it is suppressed until all the other available tactics for survival have been tried. Sporulation induction must be tightly regulated in order to be beneficial for *Bacillus subtilis* and to remain conserved through evolution.

A number of genes have been identified that are involved in the control of sporulation. The products of two of these genes are important in the overall control of the onset of sporulation: Spo0A, which is involved in sporulation initiation and the repression of transition state functions (described in section 1.8), and AbrB, which is responsible for maintaining cells in the transition state by negatively regulating the onset of sporulation, partly through negative regulation of genes whose products are involved in the transcription of *spo0A* (Hoch, 1993).

1.2. Expression of specific genes leads to morphological changes during spore development

Bacillus subtilis sporulation can be divided into a number of stages which can be seen microscopically as changes in the cell morphology (Errington, 1993). Vegetative growth is considered to be stage zero. Stage I is not distinct from stage 0, therefore all changes before stage II are considered to belong to stage 0 (Piggot and Coote, 1976). The formation of an asymmetric

septum occurs at stage II. Engulfment of the endospore by the mother cell occurs during stage II, with stage III beginning when the prespore is fully engulfed. During stage IV the spore cortex (cell wall) forms, and in stage V the spore coat is formed. Spore maturation involves with the formation of products that allow it to be dormant and resistant to environmental extremes, then germinate in favourable circumstances (Errington, 1993). This process occurs in stage VI. The final stage, when the mother cell lyses and releases the mature spore, is stage VII. Each change in cell morphology is preceded by the expression of a number of sporulation specific genes in the spore or the mother cell. Mutations in any of the genes that lead to a certain point in spore development will prevent the cell from reaching that morphological point, therefore the genes are named according to the stage where sporulation is stopped.

1.3. Control of sporulation: the sigma factor cascade

RNA polymerase is known to change its specificity for different promoter regions when it associates with different sigma factors (Helmann and Chamberlin, 1988). The timing of the different stages of sporulation is controlled by a cascade of sigma factors (Stragier and Losick, 1990). The expression of each sigma factor depends on the expression of genes transcribed by the previous sigma factor (Stragier and Losick, 1990). There are five known sporulation specific sigma factors: σ^H, which is expressed early in sporulation and is required for the expression of a number of stage 0, stage II and transition state genes, followed by σ^F and σ^E expression in stages II and III. Activity associated with σ^E is found only in the mother cell, and σ^F is active in the forespore (Stragier, 1991). Sigma G is produced exclusively in the forespore, appearing in stage III, and σ^K is specific to the mother cell, with activity seen in stages IV-V. (Stragier, 1991, Stragier and Losick, 1990). The

expression of different sigma factors, especially those involved in the later stages of spore development, also depends on the cell being at the appropriate stage in development. For instance, σ^E is expressed in an inactive precursor form, becoming active only after the mother cell has segregated from the spore (Stragier, 1991).

Other situations have been described in which different sigma factors are involved in the transcription of specific genes. In *E. coli*, a number of different σ subunits have been found that direct RNA polymerase to transcribe different sets of stress response genes, such as the heat shock protein genes transcribed by RNA polymerase with the *rpoH* gene product (σ^H), and the nitrogen fixation genes transcribed by RNA polymerase with the *rpoN* gene product (Ishihama, 1993).

1.4. Regulation of sporulation initiation

The *spo0A* gene is transcribed at low levels by RNA polymerase containing the major vegetative sigma factor in *B. subtilis*, σ^A (Chibazakura et al., 1991). Sporulation initiation is brought about by extracellular and cell cycle signals which lead to the phosphorylation of Spo0A (Burbulys et al., 1991, Grossman and Losick, 1988, Errington, 1993). These signals have not yet been characterized, however, it is known that sporulation is repressed in the presence of glucose (Chibazakura et al., 1991, Errington, 1993), requires a specific extracellular factor that has not been identified (Grossman, and Losick, 1988) and only occurs during a specific window in the cell cycle when there are two copies of the genome present but the cell has not yet started to divide (Piggot and Coote, 1976). These signals lead to the activation of the phosphorelay (described below), which resembles a complex, multistep two component regulatory system (described below). It has been postulated that the extra steps in the phosphorelay allow added mechanisms for control in

order to prevent unnecessary activation of the sporulation pathway from occurring (Perego et al., 1994).

1.5. Two component regulatory systems

Two component regulatory systems have emerged as a common mechanism of control over the expression of a number of bacterial genes involved in a wide variety of functions (Russo and Silhavy, 1993). Two component regulatory systems have also been discovered in eukaryotes; DNA sequence comparisons led to the discovery of such systems in plants and yeast (Koshland, 1993). The basic idea behind these systems is that two proteins are involved: a sensor kinase that is able to sense an environmental or cellular stimulus, and a response regulator protein that, once activated by the sensor, regulates the transcription of a specific set of genes (Russo and Silhavy, 1993). Although the specific mechanism of regulation differs between promoters the principle remains the same: the sensor is stimulated, resulting in autophosphorylation, usually on a histidine residue. Phosphate is then transferred to the response regulator, generally to an aspartate residue (Russo and Silhavy, 1993). The regulator is inactivated through dephosphorylation by the sensor, itself, or by another mechanism.

A well characterized example of a two component regulatory system is EnvZ/OmpR regulation of expression of the *E. coli* outer membrane porins OmpF and OmpC. OmpF or OmpC is expressed depending on the ionic strength of the medium the cells are grown in: OmpF is a large porin expressed at low ionic concentrations, and OmpC, a smaller porin, is expressed at higher ionic concentrations. OmpF expression is repressed when OmpC expression occurs. EnvZ senses the ionic strength of the cell surroundings and interacts with OmpR, which regulates the transcription of the two porins. Regulation of OmpF transcription is found to involve a DNA loop (Huang et al., 1994). At low

levels of OmpR~P, the protein binds to a high affinity site upstream from the *ompF* promoter, activating transcription. As cellular OmpR~P increases, it binds to a low affinity site slightly downstream from the high affinity site and at another binding site 380-354bp upstream from the transcription start site. Protein:protein interactions between the OmpR~P molecules bound at the two low affinity sites form a DNA loop that blocks RNA polymerase binding (Huang et al., 1994). When *ompF* transcription is repressed, transcription of *ompC* is stimulated by OmpR~P binding to the promoter region (Huang et al., 1994). A similar DNA looping mechanism for repressing transcription will later be considered as a possible mechanism of repression of the *abrB* promoter in *B. subtilis*. by the *spo0A* gene product.

1.6. The Spo0A protein and its activities

Spo0A is an essential component of sporulation regulation in *B. subtilis*. Its production is at a point at which information regarding the nutritional and environmental status of the cell and its cell cycle position converge, and at which the control of initiation of sporulation occurs. During sporulation initiation, Spo0A~P is required to shut down the transcription of *abrB*. AbrB is a transition state regulator that is responsible for repression of transcription of sporulation specific genes and some transition state genes, and positive regulation of *hpr* transcription (Hpr is another transition state regulator, Strauch and Hoch, 1993). Spo0A~P is also responsible for activating the transcription of a number of sporulation specific genes, including *spo0H* (the σ^H sigma factor), *spoIIA* (σ^F), spoIIG (σ^E), other *spoII* genes and *isin* (inhibitor of Sin, a sporulation inhibition protein). Spo0A~P also autoregulates, both directly (Strauch et al., 1992), and through increasing transcription of *spo0H*.

There are two promoters found upstream of the *spo0A* gene. The upstream promoter is located 198 base pairs 5' and the downstream promoter is

50 base pairs 5' from the GTG start codon of *spo0A* (Chibazakura et al., 1991). The upstream promoter is transcribed by RNA polymerase associated with the major *B. subtilis* vegetative sigma factor σ^A ($E\sigma^A$), and the downstream promoter by $E\sigma^H$; the polymerase complex formed with the σ^H sigma factor (Chibazakura et al., 1991). Transcription from the vegetative promoter (Pv) is weak and provides the base level of Spo0A in the cell that is needed for initiation of the phosphorelay. Spo0A~P is able to bind to the *spo0A* promoter in such a way that transcription from Pv is blocked, while transcription from the sporulation specific promoter, Ps, is stimulated. Ps is a much stronger promoter than Pv, therefore more Spo0A is produced, which can then be phosphorylated to continue with the sporulation initiation process (Errington, 1993).

The *spo0A* sequence shows it to be related to other response regulator proteins (Ferrari et al., 1985), with an N terminal domain related to other response regulators and a unique C terminal domain presumed to be the DNA binding region (Spiegelman et al., 1995). The N and C terminals of Spo0A can be separated by trypsin digestion, and the resulting DNA binding domain purified for in vitro studies. The DNA binding domain is able to bind DNA alone (Grimsley et al., 1994). Repression of transcription at the *abrB* promoter is similar for DNA binding domain and whole protein, while activation of transcription at the *spoIIG* promoter is less than what is observed for Spo0A~P (Spiegelman et al., 1995).

A consensus binding sequence for Spo0A has been determined to be 5'TGNCGAA3', known as the "0A box" (Strauch et al., 1990). The protein itself has been shown by DNaseI protection assays to protect a varied number of base pairs at a single 0A box, depending on the promoter (Spiegelman et al., 1995). At the *spo0A* promoter there is a 16bp protection site (Strauch et al., 1993),

while at the *spo0F* 0A binding sites a region up to 40 bp is covered (Strauch et al., 1993). These results indicate that the interactions between Spo0A and DNA vary at different promoters. DNA conformation at different promoters may vary in such a way that DNaseI is less able to access some promoter regions bound by Spo0A, causing a larger protected area, or interactions with Spo0A may be different at different promoters.

1.7. The phosphorelay in *B. subtilis*

Activation of Spo0A in *B. subtilis* is the result of a chain of multiple phosphorylations known as the phosphorelay (Burbulys et al., 1991). The phosphorelay operates in the same way as a two component regulatory system, except that there are multiple phosphotransfers between the sensor proteins, intermediate proteins and the ultimate response regulator. At this time there are three known sensor proteins: KinA, KinB and KinC. KinA and KinB are a part of the phosphorelay. The function of KinC is not known. Phosphate is transferred to Spo0A via the Spo0F and Spo0B proteins.

KinA is a cytoplasmic protein that appears to be responsible for most phosphorelay initiation. Mutants in *kinA* are able to sporulate, but at a reduced frequency (Trach and Hoch, 1993). KinA has been shown to phosphorylate Spo0F in vitro (Burbulys et al., 1991). The KinB protein has a similar carboxyl terminus to KinA but also has six potential membrane spanning regions and is therefore thought to be a membrane protein. KinB phosphorylation has not been observed in vitro due to the difficulty in reconstituting a membrane protein in a functional form (Hoch, personal communication). Mutants in *kinB* sporulate normally, however sporulation in *kinA-kinB-* double mutants is not observed (Trach and Hoch, 1993), therefore KinB must be responsible for some low level stimulation of sporulation. Extracellular and intracellular conditions are known to affect the initiation of sporulation (Errington, 1993),

therefore it is possible that KinA and KinB are the only sensor proteins required for sporulation to occur. The presence of at least two genes responsible for initiating sporulation indicates the importance of maintaining the ability to sporulate in adverse conditions to *Bacillus*.

Phosphate is transferred from KinA (and probably KinB) to Spo0F. The structure of Spo0F is typical of the N terminal portion of a response regulator (Burbulys et al., 1991, Trach et al., 1985). Spo0B acts as a phosphotransferase, transferring phosphate from Spo0F to Spo0A. This phosphotransfer reaction is reversible (Burbulys et al., 1991). Spo0A is another response regulator protein, and is the central protein involved in regulating sporulation initiation.

The multiple steps of the phosphorelay allow additional levels of control to be applied to Spo0A activation. Three proteins have been identified which decrease the amount of Spo0A~P in the cell by acting directly on the phosphorelay: Spo0E, Spo0L and Spo0P. Spo0E dephosphorylates Spo0F (Ohlsen et al., 1994). Spo0L and Spo0P are both Spo0F phosphatases. The production of Spo0L is triggered by the ComA/ComP two component regulatory system, which activates the transcription of competence genes, while Spo0P appears to be regulated by AbrB (Perego et al., 1994). Multiple levels of control over the phosphorelay pathway allow cellular levels of Spo0A~P to be tightly regulated. *B. subtilis* probably spends most of its time in a natural environment teetering on the brink between starvation and sporulation. Tight control of Spo0A~P formation, which controls the initiation of sporulation, is therefore exceedingly important in order to prevent inappropriate sporulation and yet al.low a quick entry into the sporulation pathway if all sources of nutrients are depleted.

1.8. The *abrB* gene product and transition state regulators

At the end of exponential growth, *Bacillus* enters a state in which it is no longer able to grow and divide logarithmically due to nutrient limitation, and it has evolved a number of survival strategies which come into effect at this period in growth. Genes which are necessary for the expression of the survival strategy mechanisms are known as "transition state regulators" (Perego et al., 1988). Three of these regulators are AbrB, Hpr, and Sin. AbrB directly stimulates the transcription of *hpr*, and indirectly activates Sin activity by repressing the transcription of *isin*, a gene whose product is responsible for negatively regulating *sin*. These transition state regulators activate genes that might increase the survival ability of the cell under nutrient limiting conditions. The regulated genes encode enzymes that are able to scavenge complex nutrients, such as proteases, amylase and nitrogen utilization enzymes, flagellar components for cell motility, antibiotic production in order to be better able to compete with other cell types, and bacterial competence (Strauch and Hoch, 1993). Sporulation is repressed until other avenues for cell survival are no longer effective.

AbrB is a 96 amino acid (10,500 Da, Strauch et al. 1989a) protein with a helix turn helix DNA binding motif (Perego et al., 1988). In vitro studies (Strauch et al., 1989b) have shown that AbrB protects DNA in the promoter regions of some genes that it controls. AbrB is able to have both positive and negative effects, on gene transcription. The *hut* operon produces histidase, and its transcription is activated by AbrB. DNaseI protection assays show that AbrB binds to a downstream region of the *hutP* gene, covering a hut operator site. It is probable that this binding prevents repression of *hut* transcription by another protein, possibly by preventing DNA looping by a catabolite repressor (Fisher et al., 1994). The mechanism of this repression is not known.

Negative regulation by AbrB occurs at a number of promoters. AbrB was shown by gel retardation to bind to *aprE* (the subtilisin gene), *spo0E*, *hpr*, and *abrB* DNA (Strauch et al., 1989b). A DNA sequence specific for AbrB binding has not been found. It has been postulated that AbrB recognizes a tertiary DNA structure that occurs in its target genes rather than a specific target sequence (Strauch et al., 1989b).

AbrB is expressed in vegetatively growing cells and during the transition state after exponential growth has ceased. Because AbrB negatively regulates a number of sporulation specific genes, it is necessary for *abrB* transcription to be negatively regulated at the onset of sporulation, and this is done by the *spo0A* gene product (Strauch et al., 1990). LacZ fusion studies show that downregulation of *abrB* at the onset of sporulation requires active Spo0A (Perego et al., 1988). DNase I protection assays have shown that Spo0A and Spo0A~P bind to a specific region of the *abrB* promoter in vitro (Trach et al., 1991). The Spo0A binding domain has been shown to repress transcription from *abrB* (Spiegelman et al., 1995).

1.8. Purpose of the thesis

This work was done to elucidate the mechanism by which Spo0A represses transcription from the *abrB* promoter. The structure of the promoter (Figure 1) is such that three possible models for repression were suggested (Figure 2); each model was tested. The first model is the DNA looping model. The concept is that the upstream 0A box (-213 to -218, Hoch, personal communication) and the two downstream 0A boxes found at +11 to +17 and +21 to +27 (Perego et al., 1988) all bind Spo0A, which is then able to oligomerize in such a way that a DNA loop is formed between the two sites, either blocking polymerase binding or somehow preventing the polymerase from transcribing even if it is able to bind. The model does not differentiate

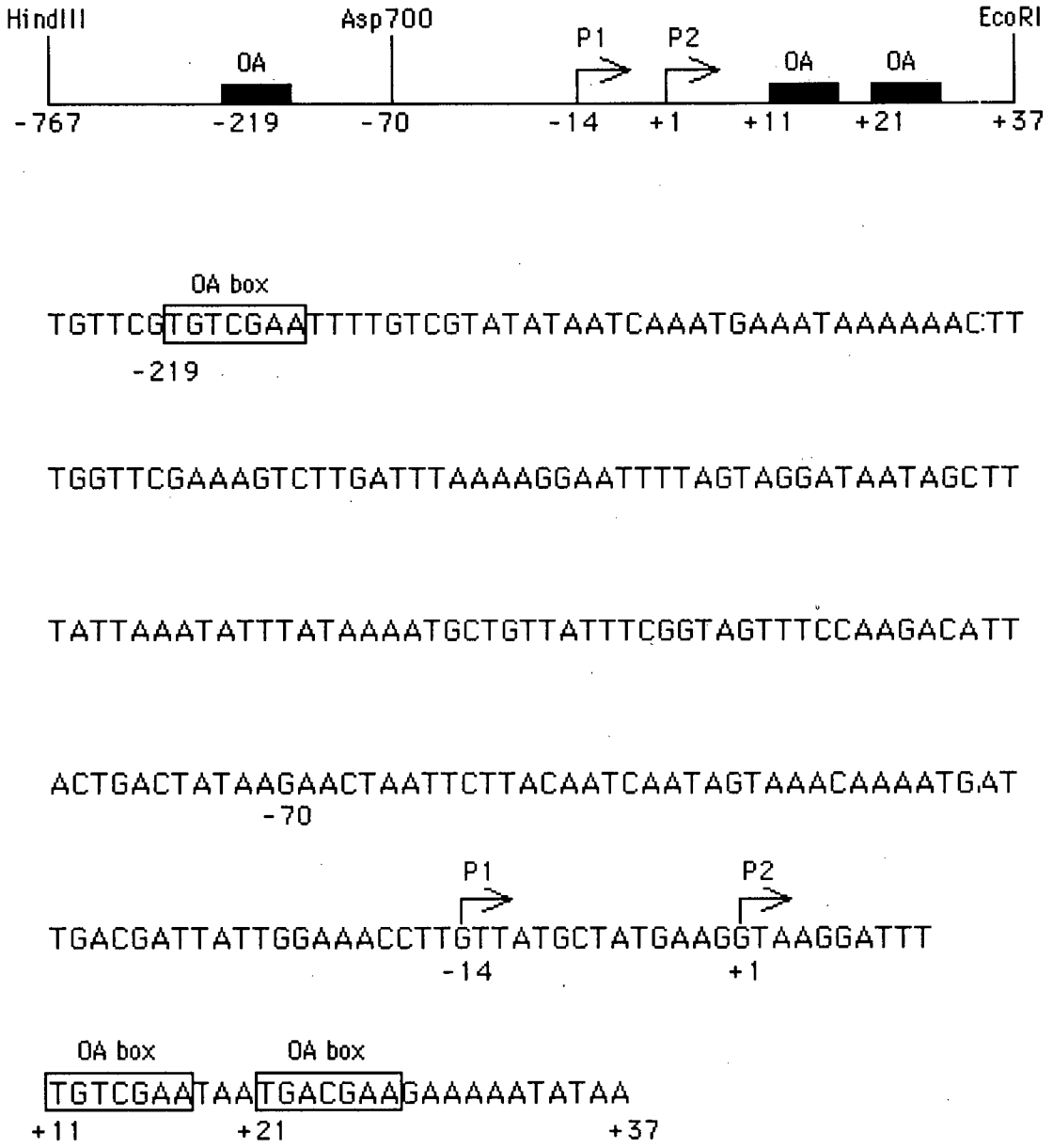

Figure 1. Structure of the *abrB* promoter region on the EcoRI/HindIII restriction fragment excised from pJM5134 plasmid DNA. The transcription start site from the P2 promoter is labelled +1 and used as a reference for the location of other sites. Bent arrows indicate promoter start sites. Spo0A binding consensus sequences are marked as "0A boxes".

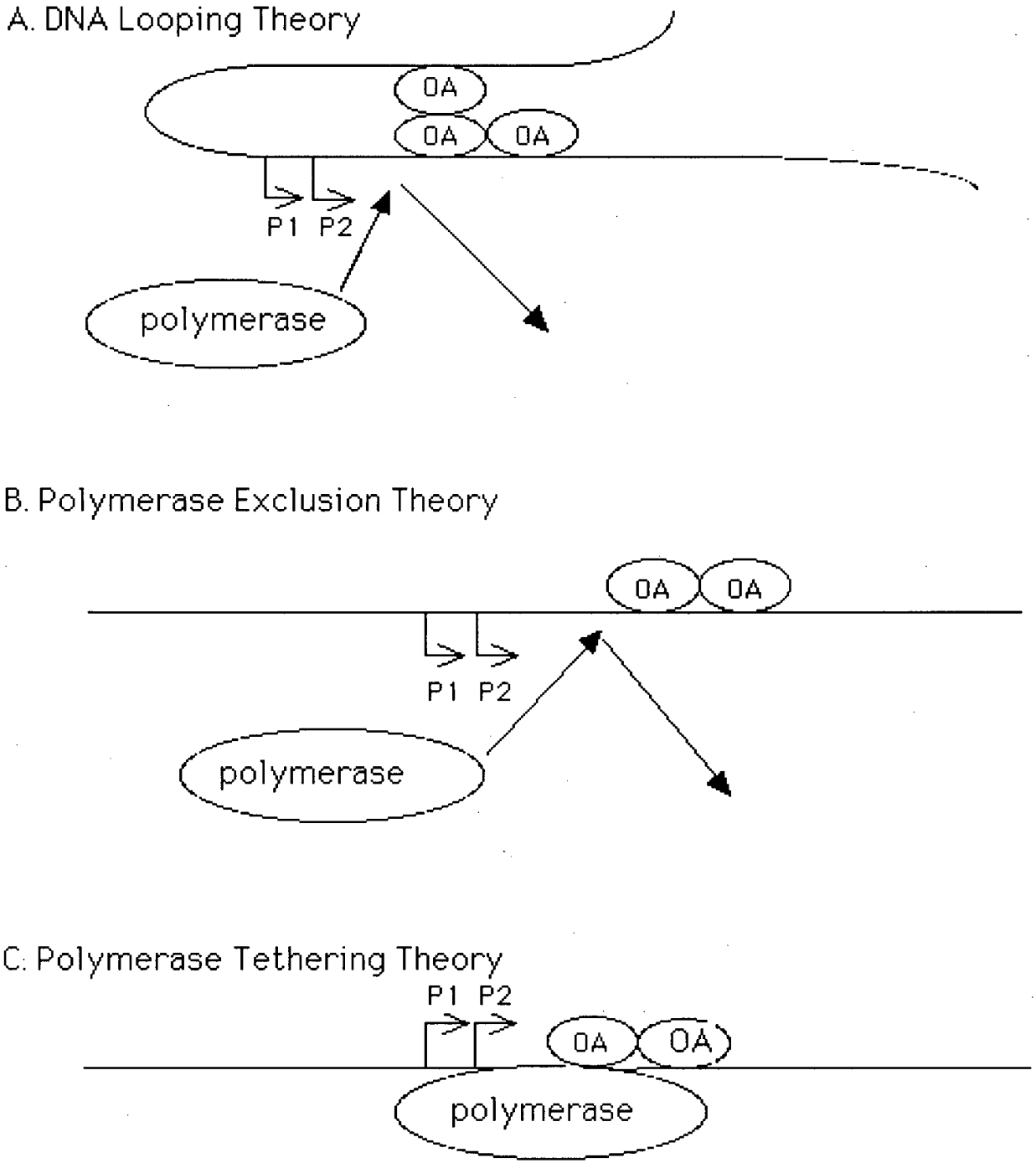

Figure 2. Three models for Spo0A(~P) repression of transcription from the *abrB* P1 and P2 promoters. Spo0A(~P) proteins are labelled "0A". Promoters are labelled P1 and P2. DNA is depicted by an unbroken line.

between these two possibilities. DNA looping to prevent polymerase binding is seen in the repression of transcription from *ompF* (discussed above, section 1.5). DNA looping is also observed as a mechanism of control by the *lac* repressor (Matthews, 1992). Repressor protein binds operator sequences at -92, +1 and +401 relative to *lacZ*. Repressor binding to the +1 operator site blocks transcription of the *lac* operon. Repressor bound to the +401 site is able to stabilize RNA polymerase binding to the *lac* promoter region as well as inhibiting *lac* operon transcription. All three operator sites are involved in DNA loop formation (Matthews, 1992). The Spo0A(~P) DNA looping model was tested by in vitro_transcription assays. If transcription repression by Spo0A~P were the same on a DNA fragment containing the upstream 0A box and one in which this site has been removed, then the upstream 0A box would not be important in the regulation of this promoter, and DNA looping would not be the mechanism by which *abrB* transcription is repressed. The DNA fragments that were used for transcription were the EcoRI/HindIII and EcoRI/Asp700 fragments indicated in Figure 1.

The second model investigated by this work is the "blocking model". Blocking of RNA polymerase binding to a promoter region to prevent transcription has been documented previously in repression of *trp* operon transcription and λ bacteriophage cI blocking of transcription of phage genes. Bacteriophage λ cI represor binds to three operator regions on the DNA. Three operator sites affect transcription from two promoters facing in opposite directions (left, P_{RM}, and right, P_R). Repressor binds first to the two right operator regions, blocking transcription from P_R and increasing RNA polymerase binding to P_{RM}. At high repressor concentrations, the left operator site is also bound and transcription from both promoters is blocked (Hochschild et al., 1983). The *E. coli trp* repressor binds to the operator region

at the *trp* promoter and prevents RNA polymerase from binding. Prebound RNA polymerase is able to block *trp* repressor binding. Repressor complexes with DNA dissociate easily, therefore a high concentration of repressor protein is required in the cell to affect *trp* operon transcription (Niedhart et al., 1987). In this thesis the blocking model suggests that Spo0A or Spo0A~P binds to the 0A boxes downstream from *abrB* P1 and P2 promoters, blocking the promoter region and preventing interaction between RNA polymerase and DNA. The upstream 0A box is not important for this mechanism of regulation, or for the third proposed regulatory mechanism, which is the tethering model.

S. B. Straney and D. M. Crothers (1987) presented a polymerase tethering model at the *E. coli lac* UV5 promoter. The authors show that *lac* repressor prevented open complex formation, but increased RNA polymerase binding to the promoter by over 100 fold. This system allows for the repression of the *lac* operon while preparing for a quick response to induction of *lac* operon transcription; upon dissociation of the repressor, RNA polymerase is already bound to the promoter. In the tethering model for inhibition of *abrB* transcription, Spo0A and polymerase are able to bind to the *abrB* promoter region simultaneously, and Spo0A is able to block transcription by "tethering" RNA polymerase. It is possible to differentiate between the blocking and tethering theories using DNA protection assays; polymerase and Spo0A(~P) protection of DNA from nuclease digestion or chemical modification should occur on the same DNA strand in the case of the tethering model, or Spo0A binding should be able to reduce (and at higher concentrations eradicate) polymerase protection of *abrB* DNA if Spo0A blocks polymerase binding. These three proposals have been tested using a combination of in vitro transcription and DNA protection assays, and a model for Spo0A repression of *abrB* transcription will be presented based on the in vitro studies.

2. Materials and Methods

2.1. Preparation of DNA

2.1.1. Isolation and preparation of *abrB* promoter fragments

Escherichia coli strain DH5α was used to produce plasmid DNA. Transformed cells were made previously by Spiegelman (University of British Columbia, Vancouver, B. C.). Plasmid pJM5134 has an 804 base pair (bp) EcoRI/HindIII fragment (Figure 1) of the *Bacillus subtilis abrB* gene and its upstream region, from -767 to +37 relative to the P2 promoter, integrated into pUC19 (Perego et al., 1988). Two litre cultures were grown for 12 hours, then 50μg/mL chloramphenicol was added and cultures left overnight to allow the plasmid to amplify (Sambrook et al., 1989). Cells were collected by centrifugation and lysed by cleared lysis (Sambrook et al., 1989). 1.13 g/mL of $CsCl_2$ and 0.5 mg/mL ethidium bromide (EtBr) were dissolved in the resulting supernatant and it was centrifuged for 36-40 hours at 147,000xg. Plasmid bands were collected and centrifuged a second time at 416,000xg for 5 hours. The resulting bands were collected, butanol extracted and the $CsCl_2$ removed by dialysis in TE. Concentration was determined through the absorbance of the sample at 260 nm, assuming that $1A_{260}$=50 μg/mL DNA. The concentration and purity of samples were checked by agarose gel electrophoresis.

2.1.2. Preparation and isolation of DNA fragments

Two fragments of plasmid DNA were prepared: an 804 base pair (bp) EcoRI/HindIII fragment and a 107 bp EcoR1/Asp700 fragment. The EcoRI/HindIII fragment was obtained by digestion of approximately 500 μg of pJM5134 DNA in 1xREact2 buffer with 30 units each of EcoRI and HindIII (BRL). EcoRI/Asp700 fragment was made by digestion of approximately 500 μg of pJM5134 with 30 units of Asp700 in 1x SurecutB buffer (Boehringer Mannheim), then ethanol precipitation of the sample (section 2.6), followed by

dissolving in 42 µL sterile water and digestion with 30 units of EcoRI in 1x REact3 buffer (BRL).

Fragments were separated by electrophoresis through a 1.2% agarose gel containing 0.2 µg/mL EtBr in 1/2x TBE buffer. The bands of interest were excised and electroeluted in 12000-14000mw dialysis tubing (Spectra/por, Spectrum Medical Industries, Inc) for 15 minutes at 6V/cm in 1/2xTBE buffer. Samples were removed from the dialysis tube and the nucleic acid was ethanol precipitated (section 2.6), resuspended in 50 to 100 µL of TE pH 7.5 and stored at $4^{O}C$. The DNA concentration was estimated by comparison of EtBr fluorescence of samples with a sample of ϕ29 bacteriophage DNA of known concentration cut with HindIII and electrophoresed through an agarose gel.

2.1.3. Preparation of end-labelled fragment for protection assays

Approximately 20 µg of pJM5134 DNA was treated with 2 units of T4 DNA ligase in 1x ligase buffer (BRL) with 1mM ATP (Pharmacia) to repair a site specific nick that occured during plasmid preparation. This nick occured in a 4 nucleotide direct repeat centred at -54. It was found on both strands at up to a 30% frequency in unligated DNA, and treatment with ligase eradicated the problem. The specific cause of the nick is unknown. Ligation was followed by ethanol precipitation (section 2.6), then the plasmid DNA was resuspended in 25 µL of sterile water and cut with EcoRI as described previously. DNA was precipitated with ethanol (section 2.6) after EcoRI digestion prior to labelling of either DNA strand.

The template strand was labelled with the Klenow fragment of DNA polymerase I. Precipitated DNA was resuspended in 1x Klenow buffer (New England Biolabs) containing 35 mM dTTP (Pharmacia), 50 µCi of α^{32}~PdATP (New England Nuclear, 3000µCi/mmole) and 6 units of Klenow enzyme (BRL). The reaction was allowed to proceed at $37^{O}C$ for 30 minutes, then the enzyme

was inactivated at 65^{O}C for 20 minutes. 10 units of HindIII (BRL), and REact2 (BRL) to a final concentration of 1x, were added to the sample and the reaction was incubated at 37^{O}C for 1-1.5 hours. The labelled fragment was isolated by electrophoresis through a 5% nondenaturing polyacrylamide gel (40% acrylamide:1.38% bis-acrylamide) at 10V/cm in 1xTBE. The bands were detected by autoradiography (30 seconds to 1 minute exposure of Kodak XAR film), excised, and electroeluted (section 2.7) in 1x TBE at 6V/cm for 1 hour. The sample was collected, ethanol precipitated (section 2.6) and the DNA pellet dissolved in 20-60 µL of TE pH7.5. 2 µL aliquots of resuspended sample were scintillation counted (Beckman LS6000IC scintillation counter) to detect the Cerenkov radiation released (counts per minute, cpm). Assuming a counting efficiency of 30%, the disintegrations per minute (dpm) were calculated and converted to moles of incorporated α^{32}P~dATP such that 1 Ci=1.2x10^{12} dpm. An assumption that 80% of the total DNA added to the labelling reaction was recovered led to a similar estimated concentration. The DNA concentration used in protection assays was approximately 2 nM.

Labelling of the nontemplate strand of the DNA duplex required removal of the 3' phosphate. EcoRI cut pJM5134 was ethanol precipitated (section 2.6), resuspended in 26 µL of sterile water and treated with 5-10 units of calf intestinal alkaline phosphatase (Boehringer Mannheim) for 30 minutes at 37^{O}C, then 7 mM EDTA and 0.1% SDS were added and the sample was incubated at 65^{O}C for 20 minutes. The sample was then extracted with 1:1 phenol:chloroform, and transferred to a sterile 1.7 mL Eppendorf tube. The phenol phase was extracted a second time with 50 µL TE pH 7.5 to collect any DNA left behind in the first extraction. The TE phase was collected and added to the DNA sample, then the sample was ethanol precipitated (section 2.6). DNA was resuspended in 1x kinase buffer (Sambrook et al., 1989), 0.6-0.8 mCi

γ^{32}P~ATP (ICN, 7000 μCi/mmole) and 10 units of T4 kinase (BRL) and incubated for 30 minutes to 1 hour at 37^{O}C, then the T4 kinase was inactivated at 65^{O}C for 20 minutes. Release of the labelled DNA fragment of interest with HindIII digestion, and isolation of the fragment were done by the same procedure as used for the template strand.

2.2 RNA polymerase purification

RNA polymerase was isolated from *Bacillus subtilis* strain 168S (Dobinson and Spiegelman, 1985). Modifications to this procedure are described in Bird (1995). The concentration of polymerase was determined by Lowry assay, comparing absorbance readings to a BSA standard. The amount of active polymerase was determined through transcription of a promoter with known characteristics using different dilutions of polymerase. The optimal polymerase concentration for transcription assays was determined for each polymerase preparation by transcription of a known quantity of DNA fragment, usually 4 nM. Dilutions of polymerase, usually 1, 1/2, 1/4, 1/8, 1/16, and 1/32, were used in transcription assays (section 2.4). The optimal RNA polymerase concentration for use in transcription assays was the lowest concentration that produced 5000 or more cpm (determined by measuring Cerenkov radiation emitted by a sample, see section 2.5 for a description of the measuring procedure). Polymerase concentrations for protection assays were determined by observing the protection from DNA cleavage provided by different concentrations of RNA polymerase (undiluted, 1/2, 1/4, and 1/8 dilutions were tested). A typical concentration of RNA polymerase used in the protection assays were two fold greater that those required for transcriptions. This is thought to be because the polymerase:DNA interactions at *abrB* are relatively unstable, and higher concentrations of protein may be necessary in

order to generate a visible level of protection than to produce easily detectible labelled transcripts.

2.3 Phosphorelay reactions

Phosphorelay reactions were carried out in 1x transcription buffer (40 mM Hepes (N-[2hydroxyethyl]piperazine-N'[2-ethanesulfonic acid]), 5 mM MgAc, 0.1 mM EDTA, 0.1 mM dithiothreitol (DTT), 0.1 mg/mL BSA pH 8.0, as described by Bird (1995), using recombinant protein stocks provided by the Hoch lab (Research Institute of Scripps Clinic, 10666 North Torrey Pines Rd., La Jolla, CA). 4 μM Spo0A was phosphorylated using 0.4 μM each of the phosphorelay proteins (KinA, Spo0F, and Spo0B. ATP (Pharmacia) was added to a final concentration of 1 mM. Reactions were incubated for 1 hour at room temperature before use. Dilutions of phosphorelay reactions were done shortly before they were used, and all samples were stored on ice after dilution. For unphosphorylated Spo0A, protein was diluted in 1x transcription buffer to a final concentration of 4 μM, and ATP was added to all initiation reactions to prevent any differences between due to the presence of ATP in the Spo0A~P sample. Addition of the other phosphorelay proteins to the Spo0A sample to prevent differences in Spo0A and Spo0A~P interactions with DNA was not deemed necessary (Bird, unpublished observations). Phosphorylation of Spo0A was followed with γ^{32}P~ATP. The results of these tests were similar to those observed by Bird et al. (1993), and indicated that the Spo0A~P was 60% phosphorylated.

2.4. Transcription assays

2.4.1. Transcription assay procedure

Transcription assays were done in 1x transcription buffer (described in section 2.3). The initiation mix contained 4 nM DNA fragment (as determined in section 2.1), 0.4 mM ATP (Pharmacia), 10 μM GTP (Pharmacia), 3 μCi α^{32}P~GTP

(New England Nuclear, 800 μCi/mmole), and 80 mM KAc, unless otherwise indicated. Reactions with UTP added to the ATP and GTP containing initiation mix contained 0.4 mM UTP. All reaction components were stored on ice. Total reaction volumes after the addition of Spo0A, RNA polymerase, and other components was 20 μL. 2 μL of Spo0A or Spo0A~P varying in concentration from 1 to 8 μM was incubated with the template for 3 minutes at 37^{O}C and the reaction started by addition of 2 μL of an RNA polymerase dilution (quantitation of RNA polymerase described in section 2.2). After an additional 3 minutes, 2 μL of 0.1 mg/mL heparin (Sigma), 0.4 mM UTP (Pharmacia) and 0.4 mM CTP (Pharmacia) solution was added and elongation allowed to continue for 5 minutes. Heparin will bind to all the free polymerase in the solution, therefore any elongation that occurs would be due to polymerase already bound to the promoter and reactions were limited to a single round of transcription (Bird, 1995). When UTP was present in the initiation mix, the elongation mix contained 0.4 mM CTP (Pharmacia) and 0.1 mg/mL heparin (Sigma). For multiple round transcription, 2 μL of a 0.4 mM UTP, 0.4 mM CTP mixture was added to the reaction and elongation allowed to proceed for a specified amount of time before 2 μL of 0.1 mg/mL heparin was added to stop initiation and the 5 minute final elongation period allowed. Reactions were stopped with 5 μL of a mixture of 10 M urea, 1/2x TBE, 0.02% bromophenol blue (BPB), 0.02% xylene cyanole (XC), then loaded directly on a 12% acrylamide gel (40% acrylamide 1.38% bisacrylamide), containing 7 M urea and 1/2x TBE, and electrophoresed at 60V/cm. PAGE was stopped when the xylene cyanole had run 8-10 centimetres. The separation achieved allowed separate quantitation of transcripts from P1 and P2. Gels were autoradiographed overnight on Kodak XRP or Kodak XAR film. Gel slices corresponding to bands on the

autoradiograph were excised and Cerenkov radiation determined to measure the relative amount of transcripts generated in each sample.

2.4.2. Determination of KAc concentration for transcription assays

Previous work showed that activation of *spoIIG* transcription by Spo0A was decreased in increasing concentrations of KAc, while Spo0A~P activation remained relatively unchanged over a range of salt concentrations (Bird, 1995). For the purpose of these experiments it was important to use a KAc concentration that allowed the differences between the effects of Spo0A and Spo0A~P to be observed. The *spoIIG* template (donated by T. Bird) was used to test the effects of salt concentration on transcription because the effects of salt concentration on this promoter have already been characterized (Bird, 1995). Transcription of 4 nM *spoIIG* in the presence of 400 nM Spo0A or Spo0A~P was carried out in 20, 40, 80, and 100 mM KAc. Reaction conditions and experimental procedures were the same as those described for *abrB* transcription (section 2.4.1). Stimulation of *spoIIG* transcription by Spo0A was approximately the same as by Spo0A~P at 20 mM KAc. 40 mM KAc caused a loss of Spo0A stimulated *spoIIG* transcription, while transcriptional stimulation by Spo0A~P remained constant to 80 mM KAc, then decreased to approximately 50% at 100 mM KAc. 80 mM KAc was therefore used in *abrB* transcription assays as it was seen to differentiate between Spo0A and Spo0A~P activity without interfering with Spo0A~P activity.

2.5. Protection assays

2.5.1. DNaseI protection assays

DNaseI protection assays were done under conditions similar to the transcription assays. The initiation mix contained approximately 100,000-150,000 cpm of labelled DNA as determined by measuring Cerenkov radiation in the labelled fragment preparations (section 2.2.3), which represents

approximately 2 nM DNA. Initiating nucleotides were 0.4 mM ATP, 0.4 mM GTP (Pharmacia), unless otherwise indicated. 0.4 mM UTP was added to the initiation mix for certain experiments. In assays where RNA polymerase was used, 2 µL of polymerase dilution buffer (10 mM Tris pH 7.9, 1 mM EDTA, 10 mM $MgCl_2$, 30 µg/mL phenylmethylsulfonylfluoride, 0.6 mM β-mercaptoethanol, 50% glycerol) was added because the glycerol content of the polymerase buffer was found to decrease DNaseI activity, and samples that did not contain polymerase were treated in the same manner as those which did whenever possible. 2 µL of 1x transcription buffer was added to all samples that did not contain Spo0A or Spo0A~P. The interactions between RNA polymerase and the *abrB* promoter were found to be weak (section 3.2.2), and background digestion of the DNA is a problem in determining protected sites at lower polymerase concentrations.

The initiation mix was warmed to 37°C, then 2 µL of 1 to 4 µM Spo0A or Spo0A~P was added and incubation continued for 3 minutes. 2 µL of RNA polymerase was then added and allowed to incubate for 3 minutes further. The total reaction volume was 20 µL. 4 µL of 10 µg/mL DNaseI was added and allowed to digest for 10 seconds, then the digestion was stopped with 5 µL of 1% SDS, 50 mM EDTA, 0.6 mg/mL yeast RNA and samples were placed on ice. Samples were precipitated by the addition of 100 µL 3 M NaAc and 750 µL 95% ethanol, incubation at -70°C for 10 minutes and centrifugation for 15 minutes at maximum speed in a microfuge, then washed with 100 µL 70% ethanol (-20°C), centrifuged for 5 minutes at maximum speed and DNA pellets were vacuum dried. DNA was then resuspended in 4 µL of 95% formamide, 20 mM EDTA, 0.5% BPB, 0.5% XC, and transferred to sterile 0.65 mL Eppendorf tubes. DNA recovery was estimated by measuring the amount of Cerenkov radiation, then samples were stored at -20°C until they were subjected to PAGE. The same quantity of

Cerenkov radiation was loaded onto each lane for PAGE. Samples were boiled for 1 minute to denature the DNA strands, then placed on ice, loaded onto an 8% acrylamide gel containing 7 M urea and 1/2x TBE and run at 50 V/cm until the xylene cyanole had migrated 30 cm. These conditions were used for the G2 and *abrB* samples. Autoradiographs were exposed for 18 to 48 hours on Kodak XAR film at -70^{O}C between two glass plates.

2.5.2. Hydroxyl radical protection assays

Initiation reactions were set up in the same manner for hydroxyl radical and DNaseI protection assays (section 2.5.1). Initiation mixture was collected in the bottom of the tube by a brief centrifugation (a 1 to 2 second pulse), then equilibrated at the reaction temperature, 37^{O}C, in a water bath. Spo0A or Spo0A~P and RNA polymerase were added and incubated as explained in the DNaseI protection assay description (section 2.5.1). Hydroxyl radical was generated by mixing 10 µL 0.2 mM FeII, 0.2 mM EDTA, 10 µL 20 mM sodium ascorbate and 10 µL 0.6% H_2O_2 on the wall of each 1.7 mL Eppendorf tube (Tullius and Dombroski, 1986) which contained the initiation mixture. FeII(EDTA) was generated by mixing a freshly prepared solution of 4 mM ammonium iron(II) sulfate hexahydrate 1:1 with 80 mM EDTA. Mixing was done in a 15 to 20 second time period before the reagents were added to the initiation mixture, with the Eppendorf tube lying horizontally across the experimenter's hand to decrease cooling of the mixture in the air. Reagent mixture was added to the initiation mix by tipping the tube vertically, then quickly closing it, vortexing briefly (1 to 2 seconds) and tapping it to settle the contents. Digestion was allowed to proceed for 1 minute in the 37^{O}C water bath, then stopped with the addition of 20 µL of a 0.1 M thiourea, 15 µg/mL sheared calf thymus DNA mixture and samples were placed on ice. The presence of glycerol was found to decrease the effects of hydroxyl radical on the samples

such that digestion times were increased to 1 minute from 10 seconds when RNA polymerase or polymerase buffer were present (this brings the glycerol concentration in the sample to 5%, section 2.5.1). An assay using samples with a 1/2 dilution of polymerase which tested the time of hydroxyl radical cleavage allowed before the reaction was stopped was done to determine the optimum incubation time for this step of the reaction; 10 and 30 second and 1, 2, and 5 minute time intervals were tested. Glycerol is known to be a hydroxyl radical scavenger (Dixon et al., 1991). Therefore, samples with polymerase or polymerase dilution buffer needed to be digested for a longer time than the original test samples, which did not have polymerase present. Precipitation was as for the DNaseI samples (section 2.5.1). Samples were resuspended in 4 µL formamide buffer, transferred to sterile 0.65 mL Eppendorf tubes and Cerenkov radiation was measured. Equal amounts of Cerenkov radiation from each sample (approximately 60,000cpm) were subjected to PAGE as for the DNaseI samples.

2.6. Ethanol precipitation of samples

3 M NaAc was added to samples to a final concentration of 0.1 M NaAc and 3 sample volumes of 95% ethanol added to the mixture. For radioactive samples, 95% ethanol was at -20^{O}C, or samples were chilled in an ethanol:dry ice bath to approximately -70^{O}C for 10 minutes; nonradioactive samples were cooled to -20^{o}C for 15 minutes to 1 hour. Differences in treatment between radioactive and nonradioactive samples were necessary because radioactive samples were kept separated from other materials in the laboratory whenever possible. DNA was precipitated by centrifugation in an Eppendorf minicentrifuge for 15 minutes at maximum speed, then washed with 100 µL of 70% ethanol at -20^{O}C and centrifuged 5 minutes at maximum speed. DNA pellets were vacuum dried and resuspended as stated in the individual experiments.

2.7. Electroelution

DNA samples were subjected to agarose gel electrophoresis or PAGE as described in the individual experiments. DNA bands in agarose gels were detected by observing fluroescence of ethidium bromide on an ultraviolet light box. Labelled DNA samples subjected to PAGE were detected by autoradiography (a 30 second to 1 minute exposure of Kodak XAR film for end labelled fragments section 2.1.3), then the bands of interest excised with sterile tools. Each gel slice was placed in a dialysis bag made from Spectrapor dialysis tubing (12,000 to 14,000 molecular weight retention cut off) with 200 to 500 µL TE pH 7.5. Dialysis tubing was laid in a horizontal electrophoresis apparatus with the gel slice toward the negative pole and subjected to electrophoresis. The buffer used was the same composition as the buffer for the gel that the slice originated from; 1/2x TBE for the agarose gel slices containing plasmid fragments for transcription assays and 1x TBE for the acrylamide gel slices containing the end labelled fragments for protection assays. Agarose gel slices were subjected to 10 to 15 minutes electrophoresis at 13 V/cm, then the charges reversed and run for 30 seconds to prevent DNA sticking to the dialysis tubing. TE containing DNA was collected from the dialysis tubing into a 1.7 mL Eppendorf tube, then the tubing was washed 1x with 100 µL TE pH 7.5, which was added to the sample. Recovery was estimated by viewing the tubing under ultraviolet light and the electroelution and washing steps repeated as necessary. Samples were ethanol precipitated as described in section 2.6, with a maximum volume of 350 µL sample per 1.7 mL Eppendorf tube, then resuspended as described in the individual experiments (section 2.1.2 for fragments for transcription assays). Acrylamide gel slices were subjected to 1 to 1.5 hours of electrophoresis at 6 V/cm, then the charges reversed for 1 minute. TE containing DNA was collected into 1.7 mL Eppendorf

tubes and 100 µL TE pH 7.5 was used to wash the dialysis tubing and added to the sample. A Geiger counter was used to quantitate the amount of radioactive material remaining in the gel slice and the dialysis tubing, and the electrophoresis and washing steps were repeated as necessary. A maximum amount of 350 µL was collected in each tube. Samples were ethanol precipitated as described in section 2.6, then resuspended as described in the individual experiments (section 2.1.3 for end-labelled fragment).

3. Results

3.1. Structure of the *abrB* and G2 promoter regions

The two promoters used for this work were from the *Bacillus subtilis* transition state regulator *abrB* (Figure 1) and the phage ϕ29 early gene G2 (Figure 3). G2 has a single promoter and is able to form heparin resistant complexes with RNA polymerase in vitro, even in the absence of initiating nucleotides (Dobinson and Spiegelman, 1987). In the following experiments, *abrB* was unable to form heparin stable complexes with RNA polymerase after formation of 1, 6 or 13 nucleotide transcripts. Interactions between RNA polymerase and *abrB* are complicated by the presence of two promoters, P1 and P2. P1 was only weakly transcribed in vitro, therefore most observations were made with respect to P2, with +1 being the first transcribed nucleotide from P2. RNA polymerase protection of G2 from hydroxyl radical attack was used to help define the characteristics of *abrB* protection under similar circumstances, because the more complex interactions at *abrB* made the polymerase protection difficult to interpret.

3.2. Characteristics of in vitro transcription from the *abrB* P1 and P2 promoters

3.2.1. Spo0A and Spo0A~P have a higher threshold for inhibition at *abrB* in single round than in multiple round transcription assays

Single round transcription from the *abrB* P1 and P2 promoters produced transcription products with less than 2000cpm of Cerenkov radiation. Methods for increasing the productivity of the promoter were explored, because higher levels of transcription and Cerenkov counts would give more accurate data. In single round transcription assays, heparin was added with elongating nucleotides. Since heparin binds to all the free polymerase but does not block elongation, only a single round of initiation is permitted. In multiple round

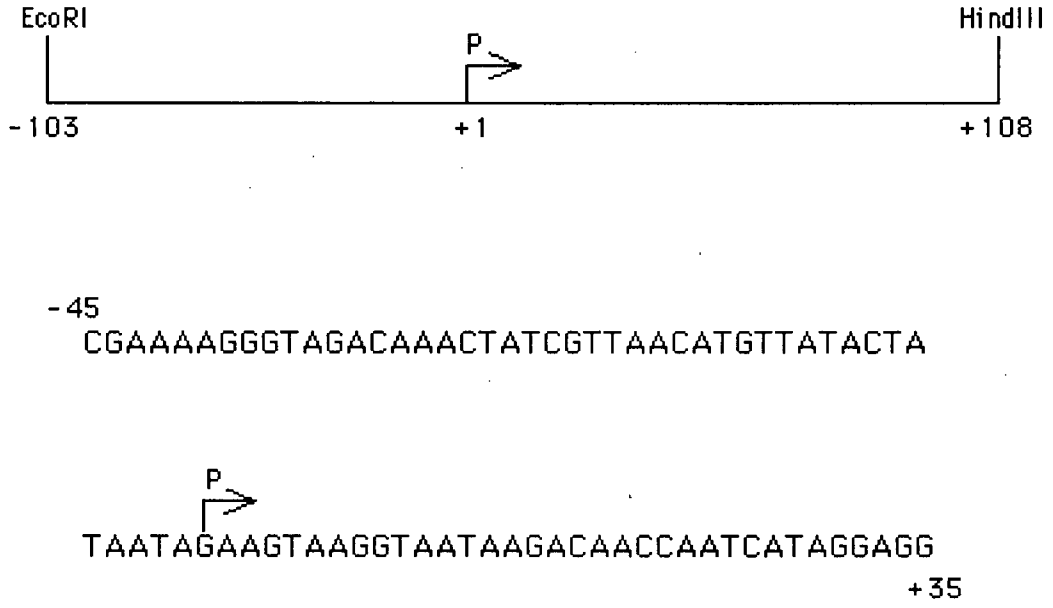

Figure 3. Structure of the G2 promoter region on the EcoRI/HindIII restriction fragment excised from ϕ29 bacteriophage DNA. The transcription start site is labelled +1 and used as a reference for the location of other sites on the DNA fragment.

transcription assays initiation is allowed to proceed for a number of minutes, thereby allowing interactions between each promoter and multiple polymerase molecules. The advantage of single round transcriptions is that it is simpler to interpret the data because only the events preceeding initiation are measured, while the advantage of multiple round transcriptions is that a higher number of transcripts are produced, therefore allowing a greater degree of accuracy in quantitating the level of transcription in different reactions.

A comparison between single and multiple round transcription was carried out. Reactions contained Spo0A or Spo0A~P at concentrations from 0 to 400 nM, 4 nM *abrB* EcoRI/HindIII DNA fragment, .4 mM ATP, .01 mM GTP, 3 μCi ^{32}P~GTP and 80 mM KAc. The products were separated on a 12% acrylamide gel, and the level of transcripts produced was determined as described in the Materials and Methods section. An autoradiogram of the gel containing products from a multiple round transcription. with the corresponding graphof the level of products are shown in Figures 4a and 4b. Spo0A and Spo0A~P both inhibited *abrB* transcription, however inhibition by Spo0A~P was two to five fold greater than inhibition by Spo0A. Figure 5 shows a comparison between single and multiple round abrB transcriptions in the presence of increasing concentrations of Spo0A~P. In single round transcription assays 50% inhibition of transcription was observed between 200 and 250 nM Spo0A~P, while in the multiple round assays 50% inhibition was observed at approximately 50 nM Spo0A~P. The difference between the two types of assay was the effective concentration of Spo0A~P; the overall inhibition curve was the same shape in either case but was shifted to a higher protein concentration in the single round transcription assays. The steepness of the repression curve suggests that cooperative binding of Spo0A and Spo0A~P to

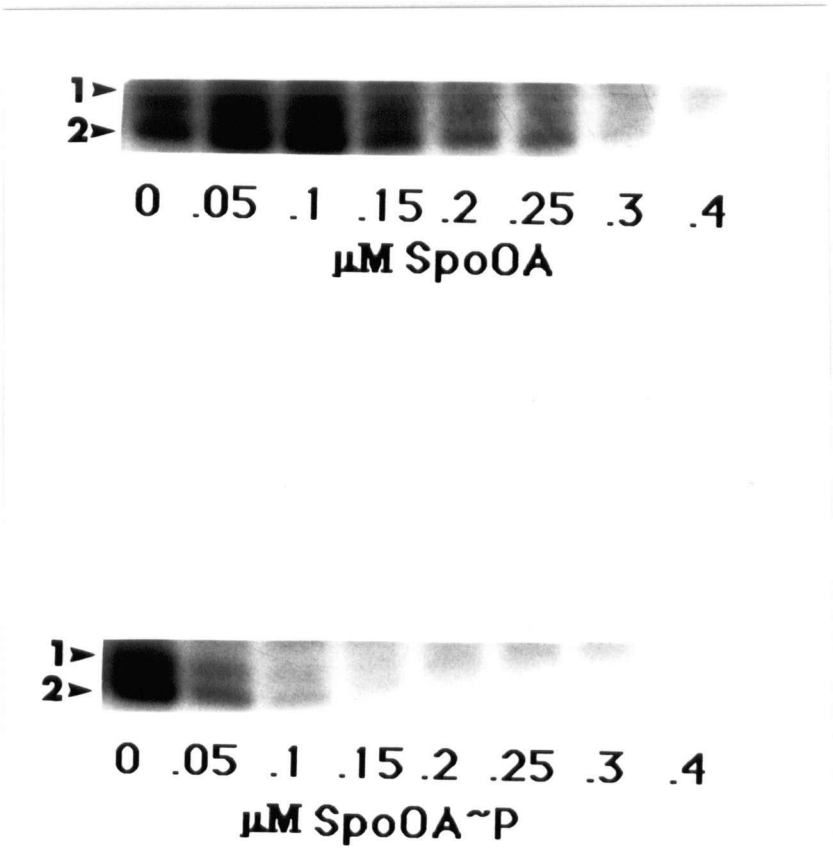

Figure 4. Effects of Spo0A or Spo0A(~P) concentration on *abrB* transcription. 4 nM DNA was incubated with the indicated concentration of Spo0A or Spo0A~P, then transcription was initiated by the addition of RNA polymerase and elongating nucleotides. Multiple rounds of transcript elongation were allowed to proceed for 5 minutes before transcript initiation was stopped by the addition of heparin.

Figure 4a. Samples were subjected to PAGE and the gel was autoradiographed. The P1 transcript (1) is 14 nucleotides longer than the P2 transcript (2) and appears above the P2 transcript on the autoradiogram. Transcription from the *abrB* P1 and P2 promoters decreased with increasing Spo0A(~P) concentration.

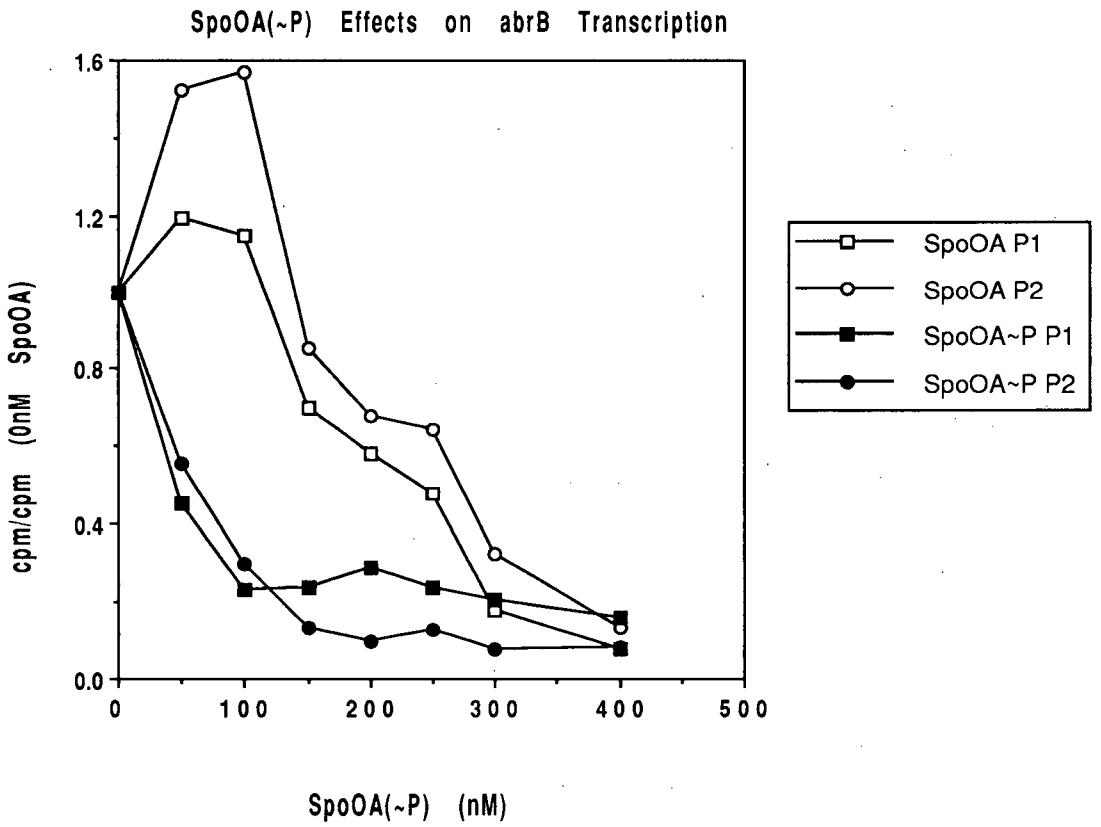

Figure 4b. Bands containing transcription products from P1 and P2 were excised and the amount of Cerenkov radiation (cpm) in each sample was determined as described in the Materials and Methods section. The cpm for each sample were normalized by dividing each value by the cpm at 0 nM Spo0A(~P) and were plotted against Spo0A(~P) concentration.

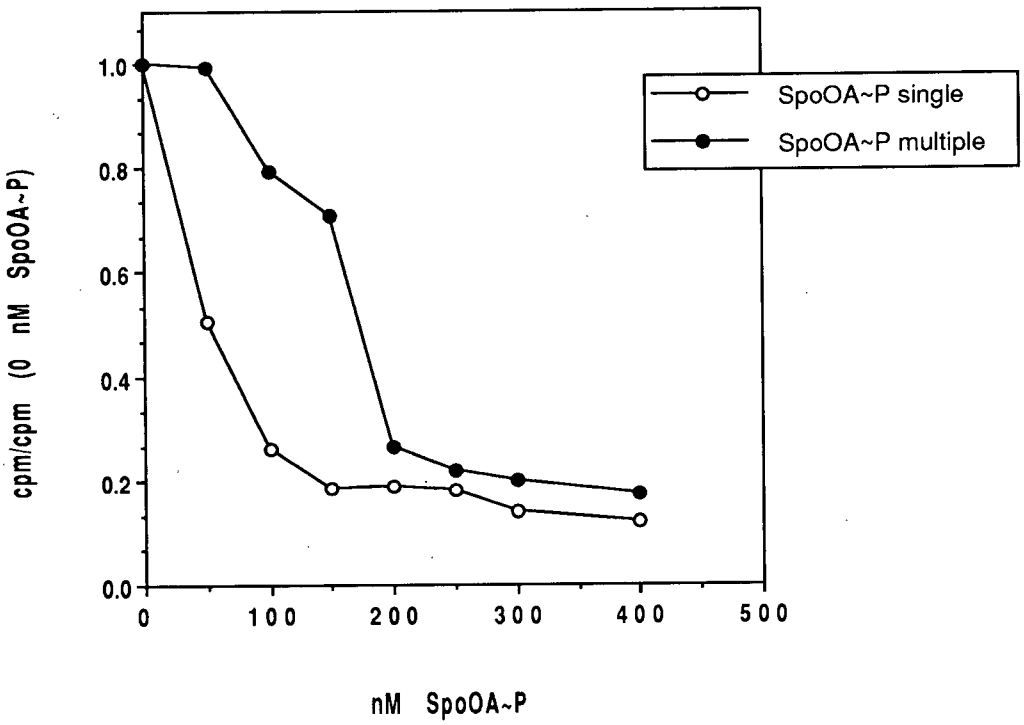

Figure 5. The effects of Spo0A(~P) concentration on *abrB* transcription in single or multiple round transcription assays. Samples were incubated with Spo0A(~P) and RNA polymerase. Heparin was added to stop further transcription initiation, at the same time as elongating nucleotides for single round transcriptions, and 5 minutes after the addition of elongaing nucleotides for multiple round transcriptions. Samples were subjected to PAGE and bands containing transcription products were excised and the amount of Cerenkov radiation (cpm) in each sample was determined as described in the Materials and Methods section. The cpm for each sample were divided by the cpm at 0 nM Spo0A(~P) to normalize the data, and plotted against Spo0A(~P) concentration.

the two downstream 0A boxes (Figure 1) occured. This possibility will be addressed in the discussion.

3.2.2. Stability of RNA polymerase:*abrB* promoter complexes to heparin challenge

The stability of RNA polymerase interactions with DNA was tested by incubating RNA polymerase with DNA in the presence of heparin for 0 to 10 minutes. RNA polymerase was incubated with *abrB* promoter for 3 minutes in the presence of initiating nucleotides ATP and GTP (producing a 1 bp transcript) or ATP, GTP, and UTP (producing a 13 bp transcript), then heparin was added and incubation continued for the specified amount of time, after which elongating nucleotides UTP and CTP (or CTP) were added and elongation allowed to occur. After one minute of heparin competition, transcription decreased to less than thirty per cent of samples where elongation was allowed to proceed immediately after heparin addition (Figure 6). If RNA polymerase were stably bound to the promoter DNA, a 10 minute incubation in the presence of heparin should have no appreciable effect on polymerase binding (Whipple and Sonenshein, 1992).

3.2.3. Differences between Spo0A and Spo0A~P preparations used in transcription and protection assays

Recombinant Spo0A was supplied by the Hoch lab (Scripps Research Institute), and a number of samples from different preparations were available. These samples were given arbitrary preparation numbers one through five. It was important to determine if different Spo0A samples had different effects on *abrB* transcription inhibition, and to choose a representative sample. To compare these samples, the activity of five different Spo0A~P preparations and two different Spo0A samples on the *abrB* promoter were tested. Reactions were carried out in 1x transcription buffer, with 4 nM

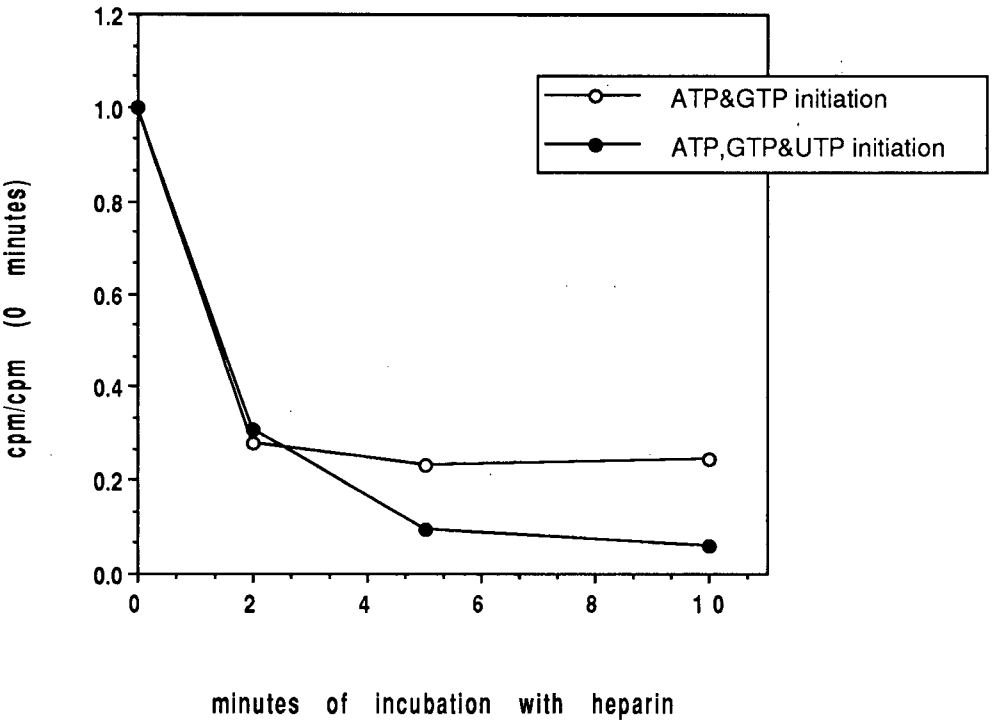

Figure 6. Heparin competition with RNA polymerase bound at the *abrB* promoter. DNA was incubated with RNA polymerase and initiating nucleotides, then heparin was added and incubation continued for the specified amount of time before the addition of elongating nucleotides. Samples were subjected to PAGE, bands containing transcription products were excised and the amount of Cerenkov radiation in each sample was determined. The values for each sample were divided by the value for the sample which had heparin and elongating nucleotides added simultaneously, and plotted against incubation time with heparin.

abrB EcoRI/HindIII promoter fragment DNA, 0.4 mM ATP, 0.01 mM GTP, 3 μCi α^{32}P~GTP and 80 mM KAc. The indicated concentration of Spo0A or Spo0A~P was added, incubated 3 minutes, then RNA polymerase added and incubated for 3 minutes before the addition of elongation nucleotides and 0.1mg/mL heparin. The level of transcripts were determined as described in the Materials and Methods section, and the results are shown in Figure 7.

It was found that all of the preparations had a similar effect on *abrB* promoter activity. Preparation 5 was the most active in repression at 200nM; preparations 1-3 stimulated transcription slightly at this concentration, however, preparation 3 was found to cause the largest inhibition of transcription at 400nM. The phenomenon of stimulation of *abrB* transcription at low concentrations of Spo0A and Spo0A~P has been observed in other experiments (Figure 8), suggesting the stimulation was representative of a general property rather than a phenomenon resulting from inconsistencies in a single protein preparation. Preparation 3 was used for all the transcription and protection assay experiments unless otherwise indicated.

Spo0A sample number 3 caused a 50% inhibition of transcription at approximately 600nM (Figure 7b), and two to five fold more Spo0A than Spo0A~P was required to repress *abrB* transcription (data not shown). Trach et al. (1991) reported (with a different Spo0A sample) that Spo0A binding required a 20 to 50 fold higher amount of protein than Spo0A~P to obtain significant protection of the *abrB* promoter region from DNaseI digestion. Protection of *abrB* DNA by Spo0A(~P) should be related to transcription inhibition, therefore it was possible that the smaller difference between phosphorylated and unphosphorylated forms was limited to Spo0A preparation 3. To test this idea, a Spo0A input was done using two other Spo0A preparations, numbers 2 and 4 (see above). Preparation number 2 had an

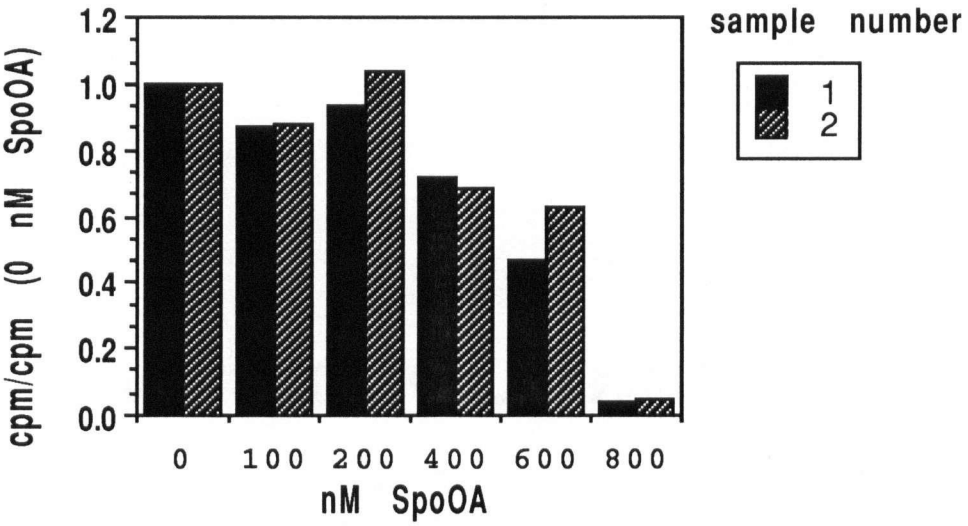

Figure 7. Differences between Spo0A preparations: Spo0A(~P) effects on transcription from the *abrB* promoter.
Figure 7a. Two concentrations (200 and 400 nM) of five different Spo0A~P samples were tested for transcription inhibition of the *abrB* promoter. Samples were subjected to PAGE and autoradiographed. The bands containing transcription products were excised and the amount of Cerenkov radiation in each sample was determined. Sample values were divided by the value obtained for control samples and graphed against Spo0A~P concentrations.

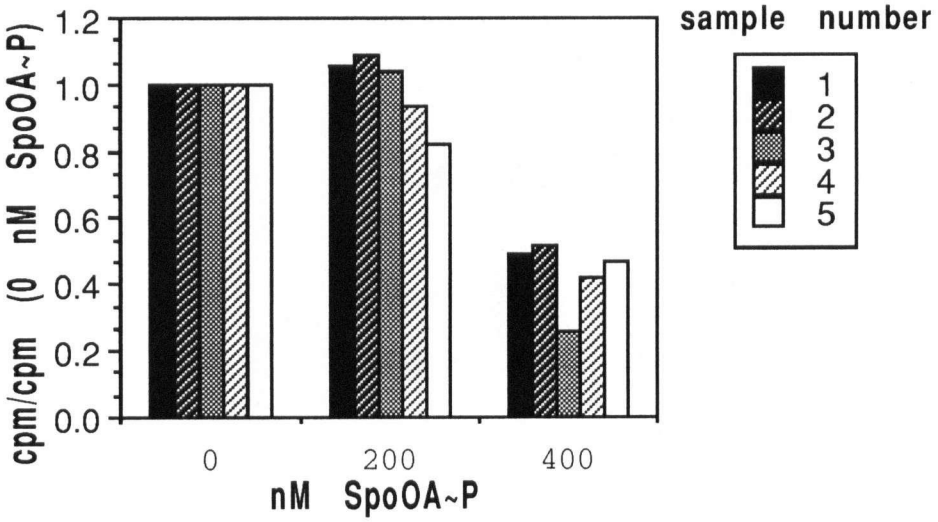

Figure 7b. Two different Spo0A samples were tested over a range of protein concentrations from 0 to 800 nM. Samples were processed in the same manner as samples in Figure 7a.

average ability to be phosphorylated via the phosphorelay reaction, and preparation number 4 had a slightly lower than average ability to be phosphorylated, while preparation 3 had a slightly higher than average ability to be phosphorylated (Spiegelman, personal communication). The results shown in Figure 7b indicate that Spo0A in preparations 2 and 4 was able to cause a decrease in *abrB* transcription such that 50% inhibition was seen at approximately 600 nM protein in both cases tested; this was a similar concentration as that observed for transcription inhibition by preparation 3 (Figure 8).

3.3. In vitro transcription of *abrB*

3.3.1. Transcription of the *abrB* EcoRI/HindIII and EcoRI/Asp700 promoter fragments is under similar control by Spo0A and Spo0A~P

The first model proposed for control of the *abrB* promoter is a DNA looping model (Figure 2). In this model, RNA polymerase is excluded from binding or from transcribing the *abrB* promoter by a DNA loop formed through interactions between Spo0A bound at the upstream and two downstream 0A boxes (see Figure 1 for the location of these boxes relative to the restriction sites used in this experiment). The most expedient method to test this model was to compare *abrB* transcription inhibition by Spo0A and Spo0A~P on DNA fragments with and without the upstream 0A box.

The pJM5134 804 base pair EcoRI/HindIII fragment represented a complete *abrB* upstream region, while the 107 base pair EcoRI/Asp700 fragment was used as the DNA fragment missing the upstream 0A box (Figure 1). One potential problem associated with this assay is that the Asp700 site is so close to the promoter region that upstream elements responsible for positive control of transcription may have been removed. A second problem is due to the upstream 0A box; Spo0A binding to this region could decrease the quantity

of Spo0A available to bind the downstream 0A boxes, thereby decreasing the inhibition of transcription of the EcoRI/HindIII promoter fragment with respect to inhibition of the EcoRI/Asp700 promoter fragment. However, in the absence of Spo0A the levels of transcripts generated using the two promoter fragments were very similar, therefore neither of these two potential problems were important for the purposes of this assay.

Figure 8 shows results from a single round transcription assay on both promoter fragments. Cerenkov counts for transcripts from both promoters in the absence of Spo0A were similar (data not shown). Spo0A was seen to cause a 50% decrease in *abrB* transcription at approximately 700 nM protein for both templates, as previously observed (Figure 7b). The 50% inhibition point for Spo0A~P in this assay was 450 nM for the EcoRI/HindIII fragment and 150 nM for the EcoRI/Asp700 fragment. There was a small amount of transcription stimulation at low protein concentrations with the EcoRI/HindIII fragment but not with the EcoRI/Asp700 promoter fragment. These results suggest that the *abrB* promoter region upstream from the Asp700 site may have some elements that stimulate transcription, or that the upstream region is responsible for decreasing the activity of Spo0A and Spo0A~P at the P1 and P2 promoters. The possibility of transcription stimulation by Spo0A at via an upstream element is supported by the slight increase in transcription from the EcoRI/HindIII promoter fragment at low levels of inhibitor. This stimulation could be mediated by RNA polymerase tethering similar to that observed at the *lac* promoter (Straney, S. B. and Crothers, D. M., 1987).

3.3.2. The effective concentration of Spo0A and Spo0A~P for 50% transcription inhibition of*abrB* is influenced by promoter DNA concentration

There was a discrepancy between the Spo0A~P concentrations which showed protection of the *abrB* promoter (Figures 10 and 11) and the

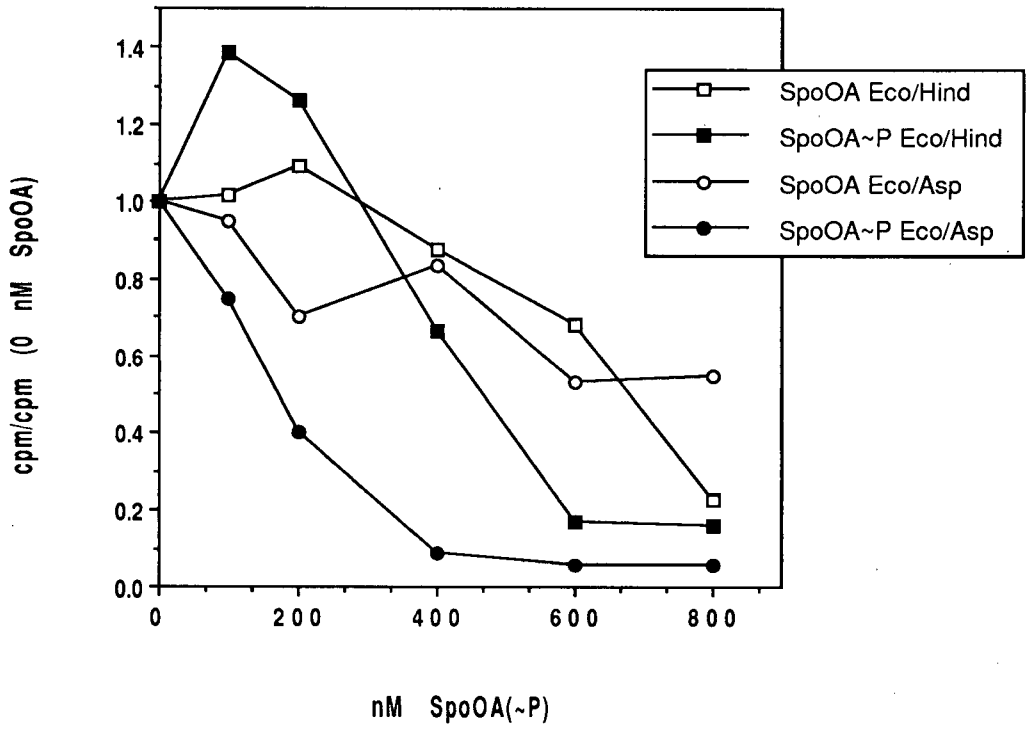

Figure 8. Spo0A(~P) effects on *abrB* transcription from EcoRI/HindIII or EcoRI/Asp700 promoter fragments. Samples were incubated with the indicated amount of Spo0A(~P), then with RNA polymerase, then heparin and elongating nucleotides were added. Samples were subjected to PAGE, autoradiographed, and bands containing transcription products were excised and the Cerenkov radiation of each sample was determined. Sample cpm were divided by the value for 0 nM Spo0A(~P) and plotted against Spo0A(~P) concentration.

concentrations of Spo0A(~P) at which transcription inhibition occurred. Spo0A(~P) appeared to protect DNA from digestion at lower concentrations than it inhibited transcription. A possible explanation for this anomaly was the difference in DNA concentration between the two different types of assay. The transcription assay contained 4 nM DNA, and the protection assays contained 2 nM DNA. To test this theory, Spo0A and Spo0A~P inhibition of transcription of the *abrB* promoter was examined at DNA concentrations from 1 to 8 nM (Figures 9a and 9b). A 40% decrease in *abrB* transcription was seen at 200 to 400 nM Spo0A and 150 nM Spo0A~P for 1 and 2 nM DNA. 4 nM DNA required approximately two fold the amount of Spo0A or Spo0A~P to show a similar decrease in transcription, and the same decrease in transcription at 8 nM DNA was shown to require four fold the concentration of Spo0A and three fold the Spo0A~P concentration required with 2 nM DNA. These results indicated that observed differences in the effective Spo0A and Spo0A~P concentrations between the transcription and protection assays were probably due to differences in DNA concentration.

3.4. Protection of the *abrB* promoter region from DNaseI digestion by Spo0A and Spo0A~P

3.4.1. Spo0A and Spo0A~P protection of the *abrB* promoter from DNaseI digestion Protection of the *abrB* promoter region by Spo0A and Spo0A~P was assayed on both the template and nontemplate strands. Inputs from 0 to 400 nM protein were used to determine the concentration at which binding began to be apparent, with the purpose of relating DNA binding activity to transcription inhibition observed in the previous sections. End-labelled DNA (2 nM) was incubated in 1x transcription buffer with 0.4 mM ATP and GTP, 80 mM KAc, and 0.4 mM UTP when indicated. Spo0A was added before RNA polymerase, and sample was incubated for 3 minutes after the addition of each

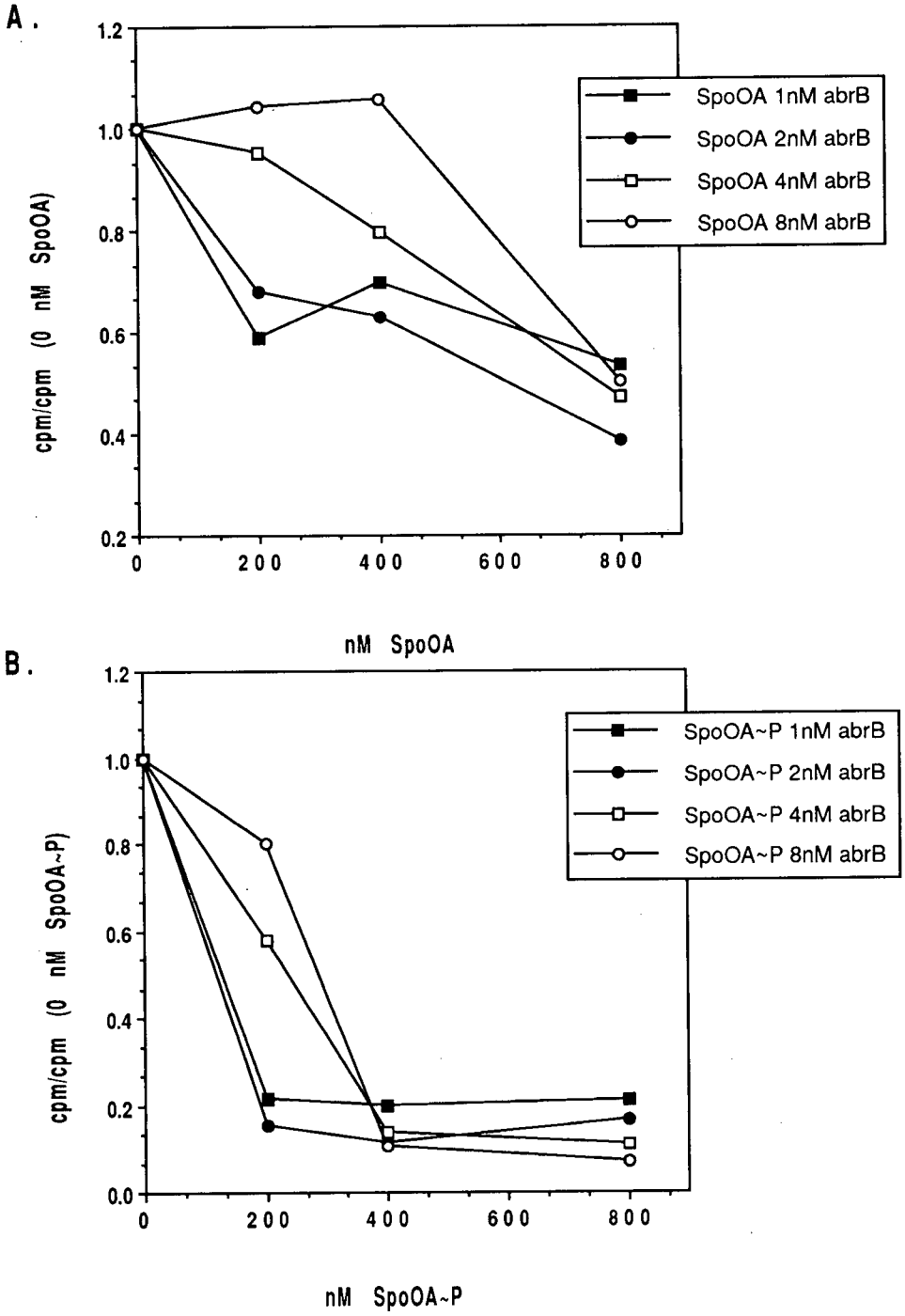

Figure 9. Effects of changes in DNA concentration on Spo0A(~P) repression of *abrB* transcription from the EcoRI/Asp700 promoter fragment. One to eight nM *abrB* template was incubated with Spo0A (A), or Spo0A~P (B), then RNA polymerase, then elongating nucleotides and heparin were added. Samples were subjected to PAGE, autoradiographed, and the bands containing transcription products were excised and scintillation counted. The Cerenkov radiation counted for each sample was divided by the values for samples containing 0 nM Spo0A(~P) and plotted against Spo0A(~P) concentration.

protein sample. All numbering of sites is relative to the *abrB* P2 promoter, which was transcribed at four fold higher levels than P1 in vitro (Figure 4a). The first transcribed nucleotide at P2 is designated 1.

3.4.1.1. Spo0A and Spo0A~P protection of the *abrB* template strand from DNaseI digestion

Spo0A binding was tested at protein concentrations ranging from 0 to 400 nM (Figure 10). Protection was seen at 400 nM Spo0A, but not at 350 nM, therefore the binding curve is sharp, possibly indicating cooperative binding at the two 0A boxes. The protected region was from +7 to +27. No hypersensitive regions were observed.

Spo0A~P protected regions on the *abrB* template strand were observed at 350 and 400 nM, but not at 300 nM protein. At 350 nM Spo0A~P protection was seen from -4 to +30, with only partial protection at -2. At 400 nM Spo0A~P protection of the -2 band was more complete, and protection extended from -38 to -15 and from -8 to +41. In a number of protection assays large portions of the DNA strand were protected at 600 to 800 nM Spo0A~P (data not shown).

3.4.1.2. Spo0A and Spo0A~P protection of the *abrB* nontemplate strand from DNaseI digestion

Binding to the *abrB* nontemplate strand was observed at a lower concentration of Spo0A than binding to the template strand (Figure 11); at 250 nM Spo0A a hypersensitive band occured at +3, which increased in intensity as protein concentration increased. Protection of the *abrB* promoter was not seen until 350 nM protein, however, which was the same as for the template strand. The protected region was from +4 to +26.

Spo0A~P was able to protect a small region on the *abrB* promoter at 300 nM (+3 to +7, Figure 11). This protected area increased in size as protein concentration increased such that at 350 nM Spo0A~P protection extended from

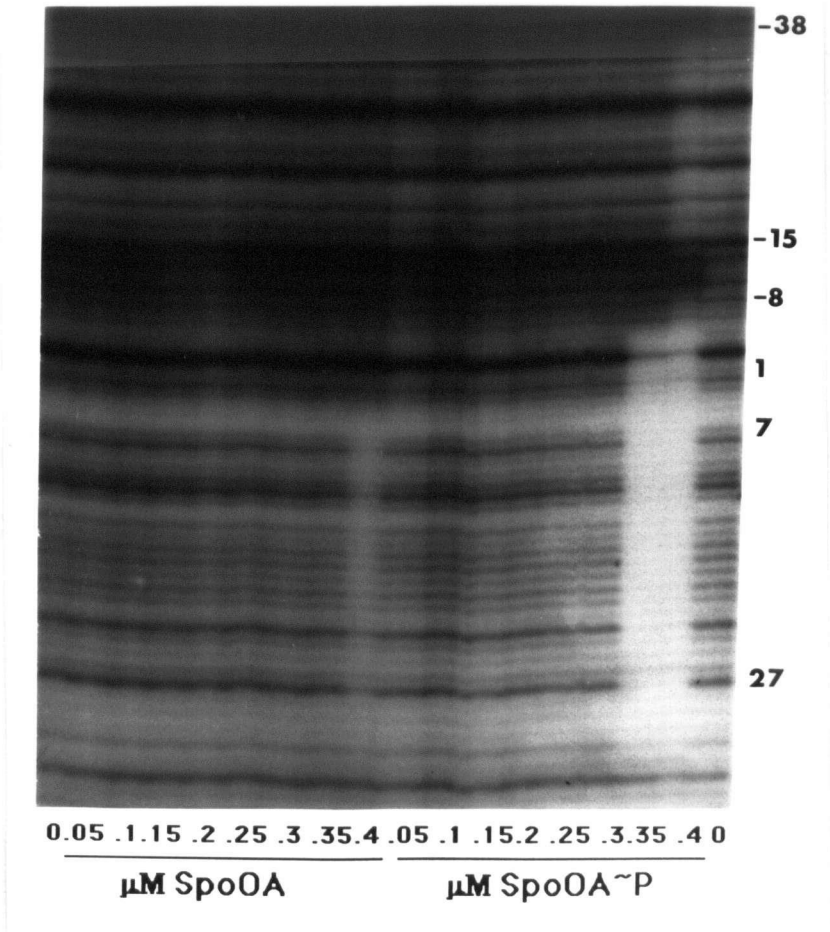

Figure 10. Spo0A(~P) concentration input on the template strand of the *abrB* EcoRI/HindIII promoter region. DNA was labelled at the EcoI restriction site with α^{32}P~dATP (3000 Ci/mmole, NEN). Spo0A(~P) interactions with the DNA were assayed by the ability of the protein to protect *abrB* from DNaseI cleavage. Samples were subjected to PAGE and autoradiographed. The position of the bands relative to the P2 promoter was determined by autoradiography of restriction endonuclease digested end-labelled DNA next to the sample lanes (data not shown), and is indicated by the numbers on the right side of the autoradiograph.

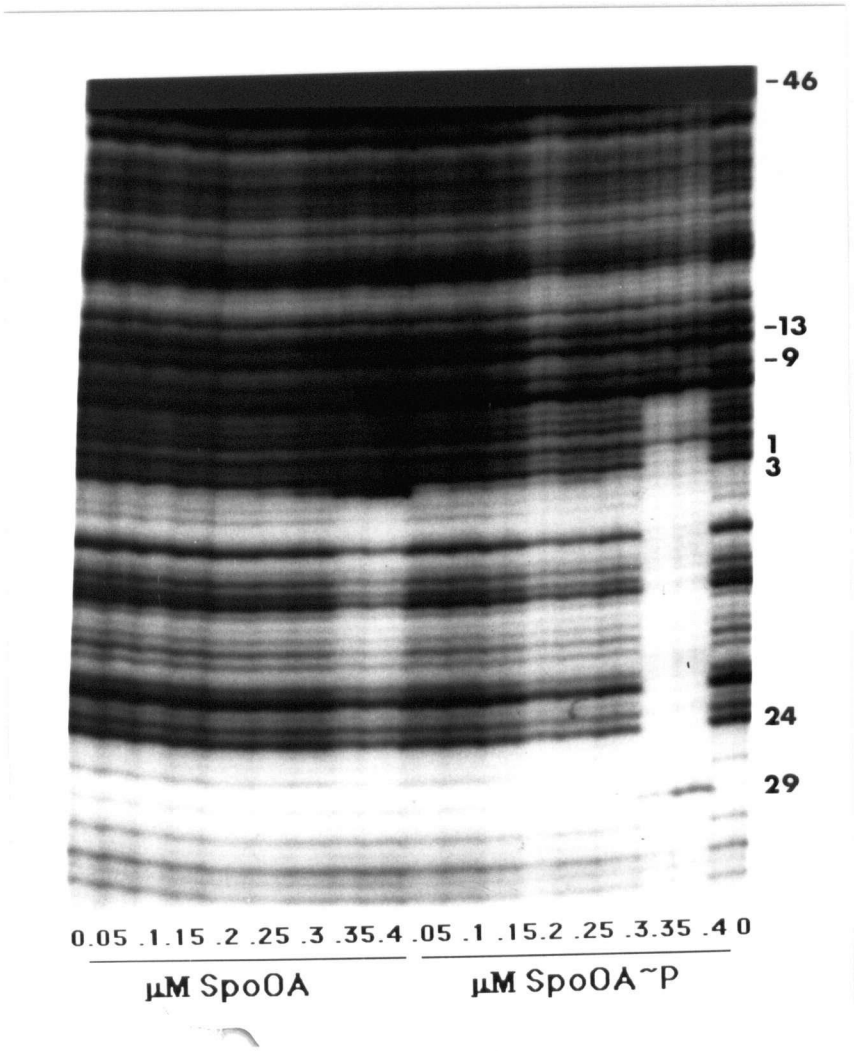

Figure 11. Spo0A(~P) concentration input on the nontemplate strand of the *abrB* EcoRI/HindIII promoter region. DNA was labelled at the EcoRI restriction site with γ^{32}P~ATP (7000 Ci/mmole, ICN). Spo0A(~P) interactions with the DNA were assayed by the ability of the protein to protect *abrB* from DNaseI cleavage. Samples were subjected to PAGE and autoradiographed. The position of the bands relative to the P2 promoter was determined by autoradiography of restriction endonuclease digested end-labelled DNA next to the sample lanes (data not shown), and is indicated by the numbers on the right side of the autoradiograph.

-4 to +42, and at 400 nM protein there was partial protection from -46 to -13, and protection from -9 to +42. A faint hypersensitive region was seen at 350 and 400 nM protein at -11, and a stronger one at +29. Low levels of the hypersensitive band observed at +3 in the Spo0A samples were present in samples with 100 to 250 nM Spo0A~P; this band disappeared at 300 nM Spo0A~P, possibly due to protection of this region by Spo0A~P at higher protein concentrations.

3.4.2. Ion effects on Spo0A and Spo0A~P protection of the *abrB* promoter from DNaseI digestion

Liermo et al. (1987) found that replacing chloride with glutamate increases interactions between protein and DNA in vitro. Chloride is not present in a free state in bacterial cells, therefore in vitro work done with glutamate is more relevant to an in vivo model for bacterial regulatory systems (Liermo et al., 1987). Acetate is similar to glutamate in its behaviour (Bird, 1995), and glutamate interferes with PAGE (Spiegelman, personal communication), therefore in this work experiments were done in KAc. Trach et al. (1991) found that in a reaction containing KCl Spo0A~P protected the *abrB* promoter region 20 to 50 fold more effectively than did Spo0A. The transcripion assays in this thesis showed that there was only a 2 to 5 fold difference in effect between Spo0A and Spo0A~P, and binding assays (section 3.4.1) showed even less difference. One candidate for the possible cause of the differences between my results and those of Trach et al. is the presence of chloride ions in the previous work. To test the effect of chloride, DNaseI protection assays on the nontemplate strand of *abrB* were done in 100 mm KAc or 100 mM KCl, with Spo0A and Spo0A~P inputs ranging from 0 to 800 nM protein (Figure 12).

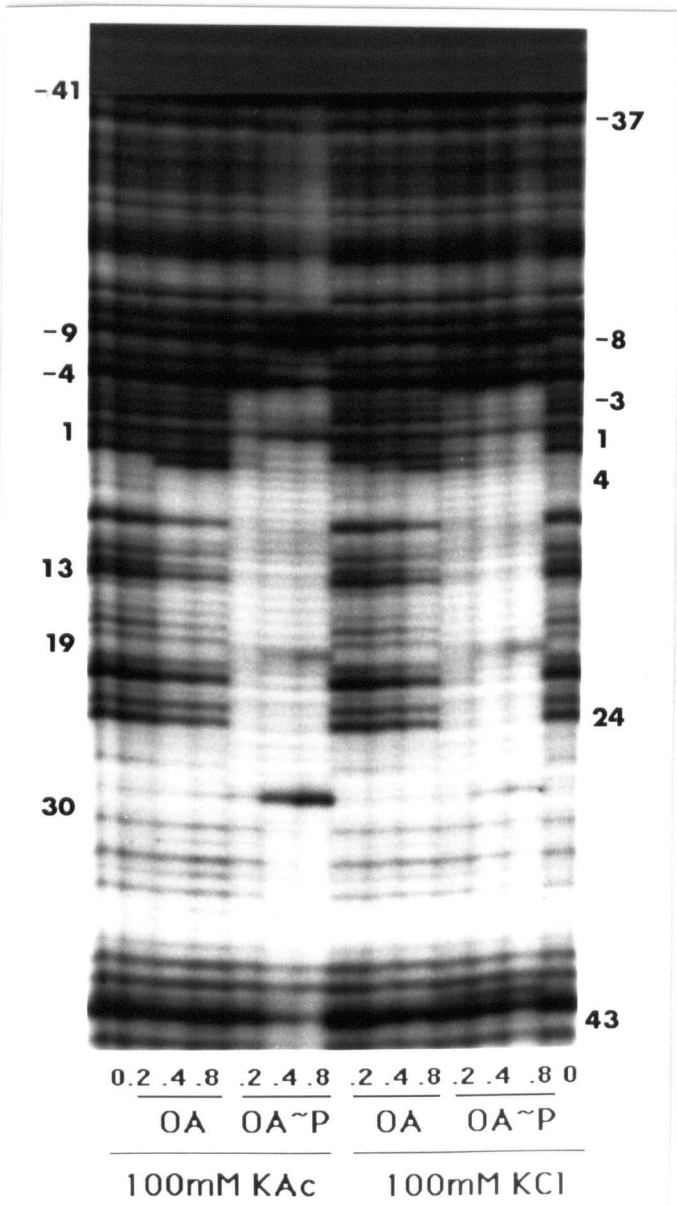

Figure 12. The effects of 100 mM KAc or KCl in the incubation reaction on the ability of Spo0A(~P) to protect the *abrB* promoter DNA from DNaseI cleavage. Spo0A(~P) was incubated with *abrB* EcoRI/HindIII promoter fragment labelled on the nontemplate strand with γ^{32}P~ARP (7000 Ci/mmole, ICN), then samples were treated with DNaseI, subjected to PAGE and autoradiographed. The position of the bands relative to the *abrB* P2 promoter was determined by autoradiography of restriction endonuclease digested end-labelled DNA next to the sample lanes (data not shown), and is indicated by the numbers beside the autoradiograph.

In the presence of 100 mM KAc, Spo0A at 400 and 800 nM partially protected a region from +4 to +27 relative to *abrB* P2 (Figure 12). A hypersensitive region at the upstream end of the protected region, +3, was observed at these concentrations of Spo0A. Spo0A~P was able to protect the same region as Spo0A, with additional protection at -41 to -13 by 800 nM Spo0A~P. An area of partial protection at 200 nM Spo0A~P was observed from -4 to +30. Partial protection only was seen from -7 to -5, and there was a hypersensitive region at +29. A faint hypersensitive region was seen at +19 in the Spo0A~P protected samples; this site was also present in the KCl assays.

In the presence of 100 mM KCl the same general regions were protected from DNaseI digestion by Spo0A as for 100 mM KAc, however, the protected and exposed bands were somewhat different, indicating that there are differences in interactions between the protein and DNA in the two different reaction conditions. Spo0A was able to protect a region from +4 to +24 at 400 and 800 nM Spo0A (Figure 12), however this protection was less pronounced than that seen with KAc. The most visible sign of Spo0A binding the *abrB* DNA was the hypersensitive band at +3, which was also less pronounced than in KAc. The hypersensitive band was visible at lower Spo0A concentrations than those needed for DNaseI protection, as observed in Figure 11. Binding in the presence of either ion occurred at a similar protein concentration. The Spo0A~P pattern in KCl was more complex than the pattern in KAc. At 800 nM protein, protection was seen from -41 to -13. This protection was only partial from -37 to -12. There was also a protected band at -5. Protection was seen from -3 to +43 with 800 nM Spo0A~P, and to +28 with 200 and 400 nM protein. There was a hypersensitive band at -8 which increased with increasing protein concentration; this band was not observed in KAc. The hypersensitive band seen at +29 in KAc was only very faintly present at 400 and 800 nM

protein in KCl, but the hypersensitive band at +19 was similar to that seen in KAc. Overall, the level of protection was lower in the KCl samples. These results showed that there may be a 2 fold difference between the binding of Spo0A and Spo0A~P in either salt condition, which does not agree with the results of Trach et al. This topic will be addressed in the discussion.

3.5. Protection of the G2 promoter by RNA polymerase

Protection assays on the G2 promoter region were done to establish a basis for comparison to interpret the results found with *abrB* protection assays. Hydroxyl radical and DNaseI protection were compared on this promoter. Hydroxyl radical attacks the sugar-phosphate backbone of DNA, therefore wherever the protein is in contact the backbone, protection will increase (Tullius et al., 1987). G2 is a strong promoter found on the bacteriophage ϕ29 (Dobinson and Spiegelman, 1985). Because it is a strong promoter, G2 is expected to show well defined regions of protection. There is only one promoter site at G2 (Figure 3), rather than the two closely linked sites seen at *abrB* (Figure 1). These factors mean that RNA polymerase protection patterns at the G2 promoter are easy to interpret, and this experiment therefore provided a good basis for interpretation of protection at the weaker and more complex *abrB* promoter sites. Reactions were done in 1x transcription buffer with 80 mM KAc and 2 nM DNA. Incubation times and digestions were done in the same manner as for *abrB* protection assays. RNA polymerase inputs under two different initiation conditions were tested on the G2 promoter: GTP initiation, which allowed elongation by a single nucleotide to occur, and AG initiation, which allowed a 4-mer to form (Figure 3). Protection assays were done on the nontemplate strand of G2. RNA polymerase concentration varied from 28 to 220 nM.

3.5.1. Protection of the G2 promoter from DNaseI digestion

RNA polymerase protection of G2 from DNaseI in GTP initiating conditions was seen from -45 to +15 (Figure 13). When ATP was added to the initiation mix, partial protection extended to +20. Hypersensitive regions were seen at -39 to -37 and -27 to -24 under both initiating conditions. With the inclusion of both ATP and GTP, protection was decreased in the -46 to -23 region and at -15. It is interesting to note that clearly defined protection was seen more at lower dilutions of polymerase; at higher concentrations it appeard that the entire DNA strand is slightly protected to some degree.

3.5.2. Protection of the G2 promoter from OH radical digestion

Protection of G2 from hydroxyl radical attack by RNA polymerase was hard to distinguish (Figure 13). Protection of the G2 promoter region from DNaseI digestion indicated that polymerase was associated with the DNA under these reaction conditions, however it may not have been tightly enough associated to prevent cleavage by the smaller hydroxyl radical, which is able to access many areas where DNaseI is blocked by size or specificity (Price and Tullius, 1992). There was partial protection of DNA from -12 to +14, particularly in the samples incubated without ATP. The notable feature of the hydroxyl radical protection was the hypersensitive regions seen from -3 to +2 under GTP initiating conditions, which extended to +6 strongly, then continued to +9 at approximately half the intensity when ATP was also added to the initiation mixture. The increase in the length of the hypersensitive region was 4 nucleotides, which was similar to the increase in length of the nascent transcript under these conditions. Similar hypersensitive regions were also observed on the *abrB* nontemplate strand in the presence of RNA polymerase (below).

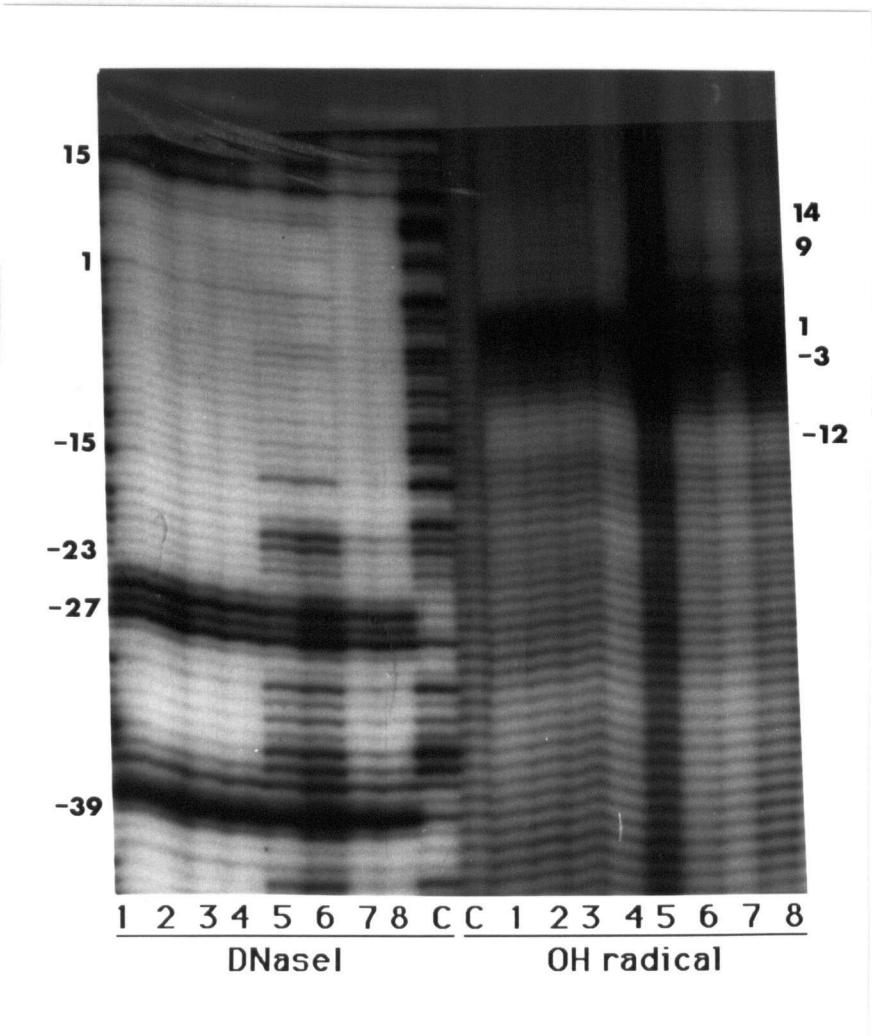

Figure 13. Protection of the G2 promoter from DNaseI or hydroxyl radical cleavage by RNA polymerase. A series of RNA polymerase dilutions were incubated with G2 EcoRI/HindIII promoter fragment labelled at the EcoRI restriction site on the nontemplate strand with α^{32}P~dATP (3000 Ci/mmole, NEN). Samples were subjected to DNaseI or hydroxyl radical cleavage, PAGE, and then autoradiographed. The lanes labelled "C" contain samples to which no protein was added. Samples in lanes 1 to 4 were incubated with GTP, and those in lanes 5 to 8 with ATP and GTP. RNA polymerase concentration was 220 nM in lanes 4 and 8, 110 nM in lanes 3 and 7, 55 nM in lanes 2 and 6 and 28 nM in lanes 1 and 5. The position of the bands with respect to the G2 promoter was determined by autoradiography of restriction endonuclease digested end-labelled DNA next to the sample lanes (data not shown), and is indicated by the numbers beside the autoradiograph.

3.6. Protection assays on the *abrB* promoter region

Protection assays on the *abrB* promoter region were done in order to determine whether RNA polymerase was able to bind the *abrB* promoter region simultaneously with Spo0A and Spo0A~P, or whether the repressor proteins were able to exclude polymerase binding. DNaseI assays were primarily used to determine the presence of proteins bound to the DNA and the region of the promoter that interacted with the proteins, while hydroxyl radical assays were used to determine specific structural changes in the DNA caused by complexes formed with proteins. Protection assays were done in 1x transcription buffer with 2 nM DNA, 0.4 mM ATP and GTP and 80 mM KAc. UTP (0.4 mM) was added when indicated. Spo 0A and RNA polymerase were added as indicated, with a 3 minute incubation period after the addition of each protein and before the next step of the reaction.

3.6.1. Protection of the *abrB* template strand from DNaseI digestion

RNA polymerase protected the template strand of *abrB* from DNaseI digestion from -64 to -59, -51 to -49, -45 to -27 and -24 to +23 (Figure 14). Protection was decreased from -51 to -49 and -45 to -27 when UTP was present in the initiation mixture and in the presence of Spo0A~P. There was a hypersensitive region at -47 and -48 with ATP and GTP initiating conditions that disappeared when Spo0A~P was added. The -2 band lost some protection by polymerase in the presence of Spo0A and Spo0A~P. The region protected by Spo0A was difficult to distinguish in this sample, however the promoter region was partially protected between +7 and +27. Spo0A~P protected a region between -5 and +31. These results corresponded with previous experiments, which showed Spo0A~P protection from -4 to +32 (Figure 11). The amount of protection of each band in these regions in both the Spo0A and Spo0A~P samples was slightly decreased when compared with the level of protection

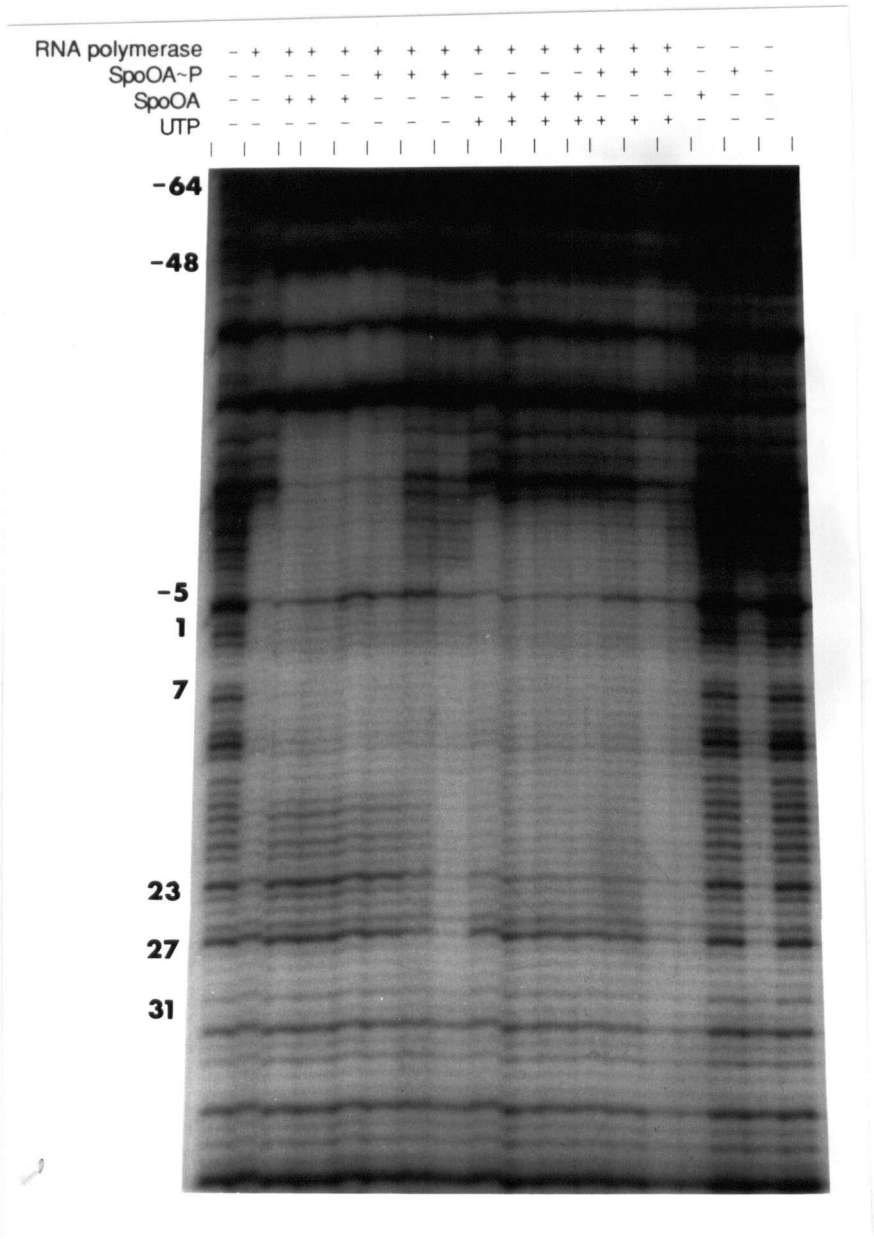

Figure 14. Protection of the *abrB* DNA template strand from DNaseI cleavage. EcoRI/HindIII DNA end-labelled with α^{32}P~dATP (3000 Ci/mmole, NEN) was incubated with ATP and GTP, and Spo0A(~P), RNA polymerase and UTP as indicated, then subjected to DNaseI cleavage, PAGE, and autoradiographed. The position of the bands relative to the P2 promoter was determined by autoradiography of restriction endonuclease digested end-labelled DNA next to the sample lanes (data not shown), and is indicated by the numbers to the left of the autoradiograph.

seen in the previous DNaseI experiment (Figure 11), but small variations between assays was common.

3.6.2. Protection of the *abrB* template strand from OH radical digestion

RNA polymerase protected a similar region of the promoter in the presence and absence of UTP in the initiating nucleotide mixture (Figure 15). Distinct protection was seen from -64 to -58, -41 to -38, -29 to -24 and -20 to +11. In the -20 to +11 region protection was decreased to partial protection from -15 to -11, -7 to -3 and +3 to +5. When UTP was present in the initiation mixture, protection extended to +20. This extended footprint was lost gradually with increasing concentrations of Spo0A~P added to the reaction. A faint hypersensitive region was visible from -4 to -1. This hypersensitive region decreased on the addition of 400 nM Spo0A, and further decreased with increasing concentrations of Spo0A~P, starting at 100 nM protein. Because Spo0A~P protected a region from +20 to +23 it was impossible to tell if polymerase still bound to this region; the polymerase:DNA interaction could only be detected to +21.

Spo0A (400 nM) protected two regions on the template strand: +10 to +12 and +20 to +22. These regions were within the area protected from DNaseI digestion (Figure 14). Spo0A~P protection of *abrB* at 400 nM protein was from -1 to +1, +9 to +13, +20 to +23, +30 to +32 and +40 to +42. The protected regions indicated that Spo0A~P binding extended to regions further downstream. Transcription assays on 2 nM DNA showed that interactions leading to protection of these downstream regions was not necessary for inhibition of *abrB* transcription since Spo0A~P inhibited transcription at 200 nM protein (Figure 9).

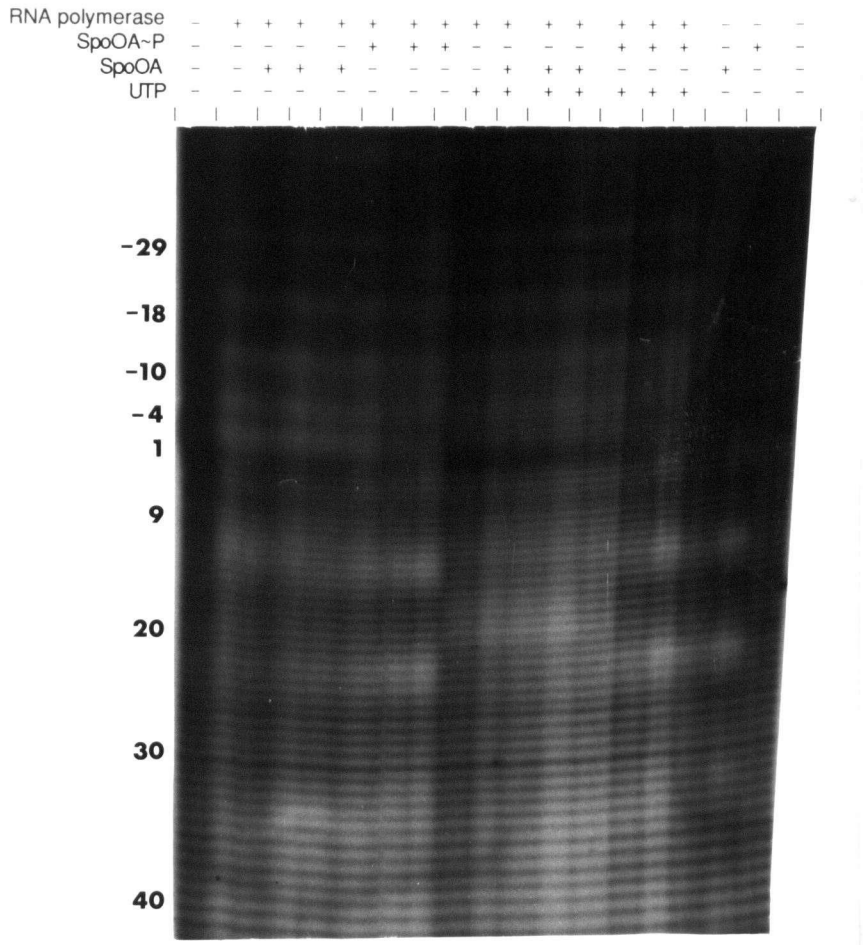

Figure 15. Protection of the *abrB* template DNA strand from hydroxyl radical cleavage. EcoRI/HindIII DNA end-labelled with α^{32}P~dATP (3000 Ci/mmole, NEN) was incubated with ATP and GTP, and Spo0A(~P), RNA polymerase and UTP as indicated, then subjected to hydroxyl radical cleavage, PAGE, and autoradiographed. The position of the bands relative to the P2 promoter was determined by autoradiography of restriction endonuclease digested end-labelled DNA next to the sample lanes (data not shown), and is indicated by the numbers to the left of the autoradiograph.

3.6.3. Protection of the *abrB* nontemplate strand from DNaseI digestion

RNA polymerase protected the *abrB* nontemplate strand from DNaseI digestion from -46 to -41 and -36 to +24 (Figure 16). A faint hypersensitive region was seen at -28, and another at -40. Spo0A partially protected a region from +8 to +19, and caused a slight hypersensitive region to form at -8. Spo0A~P protection of *abrB* was from -7 to +28, with hypersensitive bands at -8 and +29. In the presence of RNA polymerase, 400 nM Spo0A decreased protection at -8, with decreasing levels of protection as Spo0A concentration increased. Deprotection of the -8 band in the presence of RNA polymerase was seen in the presence of 400 nM Spo0A. A decrease in protection at the -8 band was more pronounced in initiating conditions that lacked UTP; however both in the presence and absence of UTP RNA polymerase was able to protect the -8 band better in the presence of Spo0A than with Spo0A~P.

3.6.4. Protection of the *abrB* nontemplate strand from OH radical digestion

RNA polymerase protected the *abrB* nontemplate strand from hydroxyl radical attack in a similar manner with and without UTP in the initiation mixture (Figure 17). Protection was seen from -46 to -42 and -31 to +20. Protection was not complete in these regions since the regions from -25 to -19 and from -17 to -12 were not protected. Deprotection was seen in the presence of Spo0A~P from +10 to +14, with protection decreasing as protein concentration increased.

When ATP and GTP were added to RNA polymerase and the abrB template, a hypersensitive region was seen from -5 to +2. By analogy with the pattern seen at G2, this hypersensitivity is proposed to indicate a region where the DNA is denatured by polymerase during initiation. When UTP was added to the reaction mixture, the hypersensitive region shifted from -5 to +2, to +3 to +10.

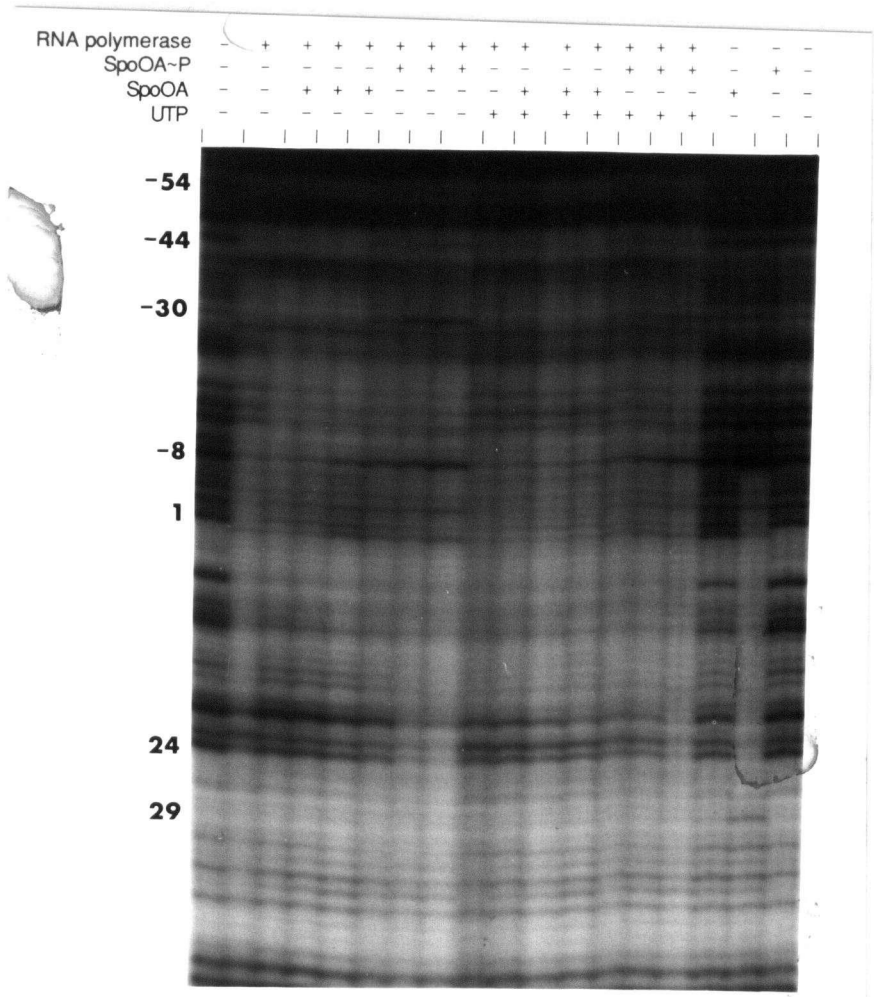

Figure 16. Protection of the *abrB* nontemplate DNA strand from DNaseI cleavage. EcoRI/HindIII DNA end-labelled with γ^{32}P~ATP (7000 Ci/mmole, ICN) was incubated with ATP and GTP, and Spo0A(~P), RNA polymerase and UTP as indicated, then subjected to DNaseI cleavage, PAGE, and autoradiogrpahed. The position of the bands relative to the P2 promoter was determined by autoradiography of restriction endonuclease digested end-labelled DNA next to the sample lanes (data not shown), and is indicated by the numbers to the left of the autoradiograph.

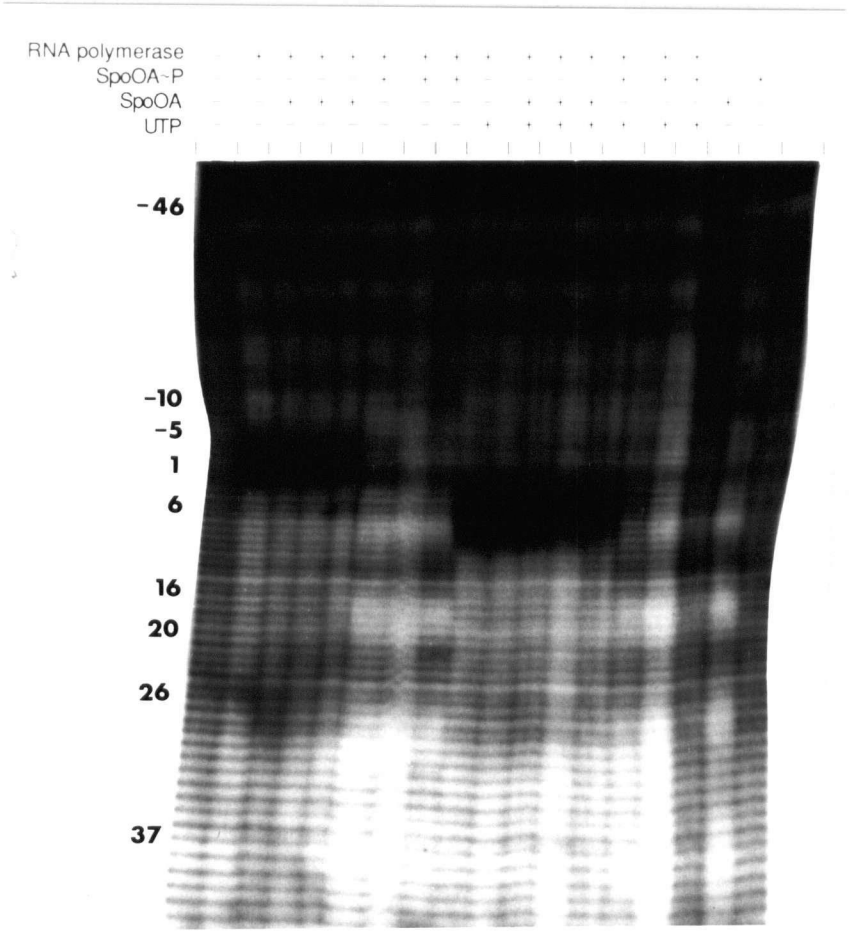

Figure 17. Protection of the *abrB* nontemplate DNA strand from hydroxyl radical cleavage. EcoRI/HindIII DNA end-labelled with γ^{32}P~ATP (7000 Ci/mmole, ICN) was incubated with ATP and GTP, and Spo0A(~P), RNA polymerase and UTP as indicated, then subjected to hydroxyl radical cleavage, PAGE, and autoradiographed. The position of the bands relative to the P2 promoter ws determined by autoradiography of restriction endonuclease digested end-labelled DNA next to the sample lanes (data not shown), and is indicated by the numbers to the left of the autoradiograph.

Spo0A protected the nontemplate DNA strand from hydroxyl radical attack between positions +7 to +10 and +17 to +20 (Figure 17). This same region was also protected from DNaseI digestion (Figure 16). Spo0A~P protected the nontemplate strand in four areas: from -7 to -1, +6 to +10, +16 to +20, +26 to +29 and +37 to +40 (Figure 17). The upstream and two downstream binding sites (-7 to -1, +26 to +29 and +37 to +40) were not observed at lower concentrations of Spo0A~P (data not shown), and protein concentrations that allowed protection of the two downstream sites were much higher than those necessary for transcription inhibition at 2 nM DNA (Figure 9). Spo0A~P created a hypersensitive band at -10 which remained in the presence of RNA polymerase with ATP and GTP in the initiation mixture but disappeared when UTP was added to the initiation mixture.

4. Discussion

4.1. Three models for transcription repression

In section 1.8., three models were proposed for regulation of *abrB* transcription by Spo0A (Figure 2). The first model required the formation of a DNA loop mediated by protein:protein interactions between Spo0A bound to the 0A boxes upstream at -219 and downstream at +11 and +27. The second model predicted that Spo0A binding to the downstream 0A boxes would exclude RNA polymerase binding to the promoter region of *abrB*. The third model was based on the possibility that RNA polymerase and Spo0A could bind to the *abrB* promoter region simultaneously. In this model, Spo0A would tether RNA polymerase at the *abrB* promoter, preventing transcription.

4.2. Mechanism of Spo0A repression of *abrB* transcription

The results obtained in this work indicate that RNA polymerase is tethered by Spo0A at the *abrB* promoter, preventing transcription. The key findings supporting this conclusion are as follows. First, transcriptional regulation of the *abrB* promoter by Spo0A and Spo0A~P occurred on *abrB* promoter fragments without the upstream 0A box (Figure 1). The proposed Spo0A mediated DNA looping between the upstream and downstream 0A boxes therefore was not the mechanism of regulation at this promoter. If *abrB* transcription had no longer been affected by Spo0A on the shortened template, DNA looping would be implicated. Secondly, in DNaseI and hydroxyl radical protection assays, RNA polymerase protection of the *abrB* promoter region was observed in the presence of Spo0A and Spo0A~P at concentrations that substantially inhibited transcription. These results indicate that Spo0A did not displace RNA polymerase from the *abrB* promoter region. The footprinting experiments argue against RNA polymerase exclusion as a mechanism for *abrB* transcription inhibition.

Spo0A and RNA polymerase bound the *abrB* promoter region simultaneously under conditions where transcription was repressed, implicating tethering of polymerase by Spo0A as a model for repression of *abrB* transcription. Inhibition of *abrB* transcription was seen between 200 nM and 600 nM Spo0A~P, with inhibition of transcription to 50% of control levels at 400 nM Spo0A~P (for the EcoRI/HindIII *abrB* promoter fragment, Figure 8). Spo0A and Spo0A~P protected the *abrB* EcoRI/HindIII promoter fragment from DNaseI cleavage at concentrations of 350 nM or higher. Spo0A interaction with the promoter region was shown at 250 nM by the presence of a band hypersensitive to DNaseI digestion at +3 (Figure 11). Simultaneous binding of RNA polymerase and Spo0A was observed in assays for protection of the *abrB* promoter region from DNaseI or hydroxyl radical cleavage (Figures 14 to 17). RNA polymerase binding was evident mainly through protection of the *abrB* promoter between -40 and -65. Spo0A and Spo0A~P protection of DNA from hydroxyl radical cleavage was evident at +10 and +20 regions of the template strand of *abrB* (Figure 15) and the +16 and +26 regions or the *abrB* nontemplate strand (Figure 17). Protection from DNaseI digestion resulted in less defined protected regions, therefore the presence of Spo0A or Spo0A~P was more evident from their effects on RNA polymerase protection between -5 and -15 on the template strand of *abrB* (Figure 14) and in the -8 and +24 regions on the *abrB* nontemplate strand (Figure 16). At these sites RNA polymerase protected *abrB* DNA from DNaseI digestion and the presence of Spo0A or Spo0A~P resulted in deprotection of the DNA but not in RNA polymerase dissociation; upstream protected regions indicated that the enzyme remained attached.

Protection of the *abrB* promoter by Spo0A, Spo0A~P and RNA polymerase from DNaseI and hydroxyl radical cleavage was never complete. There are

three possible explanations for this observation. First, background bands due to degradation of the template were evident. When untreated DNA samples were subjected to denaturing PAGE and the gel was autoradiographed, the resulting autoradiograph showed low levels of DNA degradation which increased significantly over a period of seven days (data not shown). This background effect was expected to affect protection assays involving any of the protein samples used. The second factor involves the *abrB* promoter structure. The *abrB* promoter region has two promoters separated by 14 base pairs, P1 and P2 (Figure 1). In the in vitro conditions defined for this thesis, P2 was transcribed to levels 4 fold greater than P1. As P2 was the major promoter it was expected that most interactions would occur at P2, however interactions at P1 may complicate the results by adding to background with the protection patterns observed for P2. Thirdly, RNA polymerase is not stable on the *abrB* promoter, as shown in heparin competition assays (Figure 6). It was predicted that RNA polymerase dissociated from the *abrB* promoter region during the 1 minute hydroxyl radical cleavage reactions, and possibly during the 10 second DNaseI digestions, allowing cleavage of sites protected by polymerase to occur.

Transcription inhibition to approximately 80% of control levels occurred at Spo0A protein concentrations which did not cause complete protection of the *abrB* promoter from DNaseI digestion (Figure 8). Spo0A was able to partially protect *abrB*, however complete protection was not observed with protein concentrations up to 400 nM (Figures 10 and 11). Trach et al. (1991) observed complete protection of *abrB* by Spo0A at 1.2 μM protein. Repression of *abrB* promoter transcription to background levels may have required a Spo0A concentration that would have completely protected the promoter region. This possibility was not explored. Repression of *abrB*

transcription by Spo0A~P was observed to 10% to 20% of control levels, however transcription levels never reached zero. Initiation of sporulation is a complex process that is only partially understood, and Spo0A is not the sole mediator of the initiation process (Hoch, 1993). Transcription of *abrB* is under the control of Spo0A (Perego et al., 1988), and AbrB (Strauch et al., 1989b), and may be controlled by other factors as well, therefore it is possible that other proteins are also involved in *abrB* repression in the cell.

Transcription and protection assays were conducted under similar conditions whenever possible so the results from the two assay types should be comparable. Small differences between individual assays occurred; however, the general trends in the observed results remained constant; therefore, these irregularities were considered to be minor. A consistent difference in Spo0A and Spo0A~P concentration required to produce measurable effects in the two types of assays was observed. Fifty percent inhibition of transcription required 400 to 500 nM Spo0A~P or 700 nM Spo0A (Figure 8). similarly, maximum protection of the *abrB* promoter region was clearly observed at 350 nM Spo0A~P, while 350 to 400 nM Spo0A produced only partial protection (Figures 10 and 11). Two consistent differences between the conditions of the two assays were: 1) DNA concentration, which was two fold higher in the transcription reactions than in protection assays, and 2) the presence of heparin in the transcription reactions. Heparin was added to the transcription assays at the elongation step and therefore was not expected to change the relative effects of Spo0A or Spo0A~P mediated repression of transcription from the *abrB* promoters, therefore this idea was not explored further.

The effects of DNA concentration on Spo0A repression of *abrB* transcription were tested. Fifty percent repression of *abrB* transcription was

achieved at 150 nM Spo0A~P for 1 and 2 nM template, 300 nM Spo0A~P repressed *abrB* transcription 50% at 4 nM template and 8 nM *abrB* transcription was 50% repressed at 350 nM Spo0A~P (Figure 9b). It is therefore possible that lower DNA concentrations in the protection assays were responsible for the observed differences between concentrations of Spo0A or Spo0A~P required to inhibit *abrB* transcription and those required to protect the *abrB* promoter region from DNaseI cleavage.

Tethering of RNA polymerase by Spo0A appears to be the mechanism by which Spo0A inhibits *abrB* transcription. Transcription assays were used to define the conditions under which repression occurred. Spo0A, Spo0A~P and RNA polymerase interactions with the *abrB* promoter region were defined using DNaseI and hydroxyl radical protection assays. Protection of the *abrB* promoter by both RNA polymerase and Spo0A or Spo0A~P was observed to be simultaneous. Conditions under which simultaneous protection by RNA polymerase and Spo0A occurred on the *abrB* promoter were similar to those under which transcription inhibition took place, implicating the tethering model as a viable explanation for Spo0A repression of *abrB* transcription.

4.3. Characteristics of DNA protection patterns from hydroxyl radical attack

4.3.1. Hydroxyl radical cleavage of DNA

Hydroxyl radical removes a hydrogen ion from deoxyribose sugars in the DNA backbone, cleaving the DNA molecule (Tullius et al., 1987, Tullius and Dombroski, 1985, Wu et al., 1983). The hydroxyl radical is highly reactive, and shows little specificity for sequences or bases and can be used to determine protein binding in regions where DNaseI and other types of cleavage may be incomplete (Tullius et al., 1987). A slight decrease in cleavage by hydroxyl radical can be observed in AT rich stretches of DNA sequence. The slight protection of DNA in these regions is due to a narrowing of the minor groove,

which decreases the exposure of the DNA backbone to the hydroxyl radical (Tullius et al., 1987). Drew and Travers (1984) found that the minor groove narrows considerably in AT rich stretches of DNA due to the angle at which the bases interact. A slight decrease in hydroxyl radical cleavage of the *abrB* promoter region was observed in AT rich sequences; this appears to be an almost sinusoidal pattern due to the high frequency of such sequences in the *abrB* DNA (Figures 1, 15 and 17).

Proteins binding across a minor groove in B-DNA show regions of protection on the template and nontemplate strand that are offset by three bases, while proteins binding across the major groove will show an offset of at least seven bases. Proteins bound to one side of DNA will protect each strand with a ten base pair periodicity (Tullius et al., 1987), as long as they protect more than 10 base pairs. Enhanced cleavage by the hydroxyl radical is not well understood. Enhanced cleavage was noted at one end of the region protected by KorB at the *trbB* promoter, and was observed at the same site with DNaseI protection assays (Williams et al., 1993). The authors propose that KorB bends DNA in this region, causing the observed enhancement of cleavage.

DNaseI digestion at the *abrB* promoter was not uniform since there were regions where DNA binding by RNA polymerase, Spo0A and Spo0A~P could not be determined because no cleavage occurred even in the absence of protein. Cleavage by DNaseI occurs preferentially next to pyrimidine nucleotides (Sambrook et al., 1989), therefore cleavage is limited in certain regions of DNA. DNaseI is also limited by its size; DNaseI may be unable to access regions of DNA that are not bound by protein due to steric hindrances, causing the protected region to be larger than the region of DNA interacting with the protein. This problem was observed by Sawadogo and Roeder (1985) with USF protein binding to an adenovirus major late promoter region. A 20 nucleotide region

was protected from DNaseI cleavage, while protection from methidiumpropyl-EDTA-Fe(II) cleavage was only 12 base pairs. DNaseI is also sensitive to the configuration of the DNA backbone; cleavage is most efficient if paired phosphate residues on the two opposite strands face one another across the minor groove (Drew and Travers, 1984).

4.3.2. Protection of the *abrB* promoter from hydroxyl radical attack by RNA polymerase, Spo0A and Spo0A~P

Spo0A and Spo0A~P bound to *abrB* promoter DNA at two major sites (Figures 15 and 17). The spacing of protected bases at these binding sites indicated that Spo0A and Spo0A~P bound to one side of the DNA across a minor groove. At concentrations of Spo0A~P above 400 nM, the protein also bound to two further downstream sites, each 10 bases from the previous site, indicating that the protein was still bound to the same side of the DNA. These sites covered only two to three rather than three to four nucleotides on each strand, and may have represented protection due to oligomerization of Spo0A~P on the DNA. The two binding sites furthest downstream from the promoter were only observed at concentrations of Spo0A~P that were higher than necessary for more than 80% repression of transcription from the *abrB* promoter compared to control levels (Figures 9, 15 and 17). The 0A boxes downstream from the P2 promoter are from +11 to +17 and +21 to +27. The regions protected by Spo0A and Spo0A~P in this assay were from +6 to +13 and from +20 to +23 on the template strand, and from +6 to +10 and +16 to +20 on the nontemplate strand. Spo0A(~P) protection of the *abrB* promoter from hydroxyl radical attack therefore indicated that interactions between the protein and DNA occurred in the 0A box region on the template strand but were shifted upstream from the 0A box by 4 nucleotides on the nontemplate strand. The nontemplate strand

sequences protected were CTAAA from +6 and TTATT from +16. If a consensus sequence exists for Spo0A in this region it is not obvious from these results.

RNA polymerase incubated with ATP and GTP protected regions from -64 to +11 (+20 when UTP was also present in the initiation mix) on the *abrB* template strand and -46 to +20 on the *abrB* nontemplate strand under both initiating conditions. Transcription should be able to proceed either 1 nucleotide from *abrB* P1 and P2 promoters when ATP and GTP only were present, or 6 nucleotides from P1 and 13 nucleotides from P2 when UTP was added to the initiation mixture. Addition of UTP to the initiation mixture did not change binding at the upstream sites, therefore transcription initiation proceeded by at least 13 nucleotides before RNA polymerase released its upstream binding sites. Protection by polymerase in the region upstream of the promoter between -46 and -6 appeared to be mainly on one side of the DNA; protection of the template and nontemplate strands alternated such that one side was always protected and the other exposed. The protected areas on each strand ranged from 2 to 4 nucleotides in length, indicating that protection occurred across the minor groove at each site. Metzger et al. (1989) noted that protection of the T7 A1 promoter from hydroxyl radical cleavage by *E. coli* RNA polymerase showed that the enzyme interacts with one side of the promoter DNA between -52 and -11. The DNA binding properties of *E. coli* and *B. subtilis* RNA polymerase at the T7 A1 and *abrB* promoters, respectively, appear to be similar. The regions between -6 and + 20 were protected on both strands. An interesting exception to the protection in this region is the occurrence of hypersensitive sites on the nontemplate strand (Figure 17) and the partial loss of protection and the slight hypersensitive region on the nontemplate strand (Figure 15). A possible explanation for the loss of protection and hypersensitive regions observed in the RNA polymerase protection of the

abrB promoter from hydroxyl radical cleavage is discussed below (section 4.3.3). Protection in these regions on both strands suggested that RNA polymerase was wrapped around the entire DNA helix in from -6 to +20, rather than interacting with only one side as it appeared to do in the regions further upstream. Similar observations were made by Metzger et al. (1989) for *E. coli* RNA polymerase binding between -13 and +18 on the T7 A1 promoter.

Hydroxyl radical protection assays with *E. coli* polymerase on the T7 A1 promoter did not yield the hypersensitive regions observed in this thesis (Metzger et al., 1989). The protection patterns of the two RNA polymerases were similar (as described above). Some basic interactions between *E. coli* and *B. subtilis* RNA polymerase and DNA in the melting region at each promoter must differ, however, because the hypersensitive regions that appeared in the *abrB* protection assays were not evident in the experiments by Metzger et al. A precedent for interpretation of the hydroxyl radical data in Figures 15 and 17 did not exist, and attempts at permanganate cleavage of denatured DNA to confirm this result were unsuccessful. RNA polymerase protection of the strong G2 promoter from phage ϕ29 (Dobinson and Spiegelman, 1987) was therefore used to establish a pattern for comparison with the results from the *abrB* promoter. A summary of hydroxyl radical cleavage patterns for the *abrB* promoter region is shown in Figure 18.

4.3.3. Interpretation of RNA polymerase protection of the G2 promoter from hydroxyl radical attack

Protection of the G2 promoter region on the nontemplate strand from hydroxyl radical attack by RNA polymerase was tested to determine if the hypersensitive regions around +1 on the *abrB* promoter were a phenomenon intrinsic to that promoter, or if this occurrence was common to promoters transcribed by RNA polymerase $E\sigma^A$. The G2 promoter was chosen because RNA

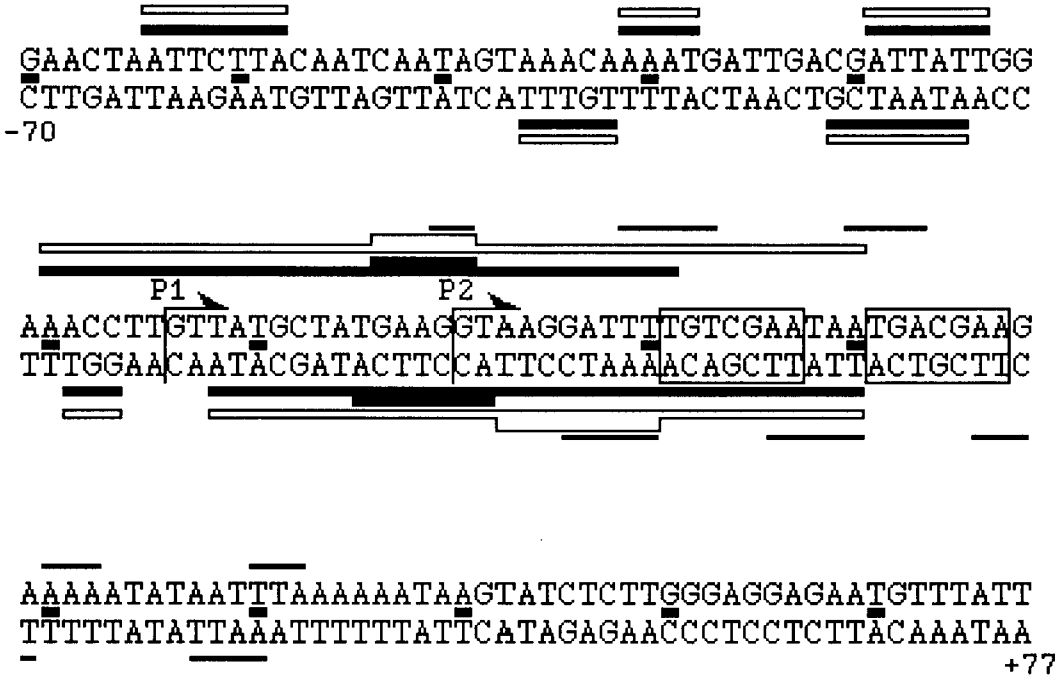

Figure 18. Summary of hydroxyl radical protection by Spo0A~P and RNA polymerase at the *sbrB* promoter. Spo0A~P protection is shown by ——. RNA polymerase protection is depicted by ▬▬ for transcription by one nucleotide, and for transcription by 13 nucleotides, by ▭. Transcript length is measured from the P2 promoter.
The promoters are indicated by ——➘, and the 0A boxes are outlined on both strands by boxes ▭. Markers (▬) are placed between the strand sequences every 10 nucleotides, starting with -70. The top strand is the template DNA sequence. The DNA sequence was provided by the Hoch lab (Scripps Institute for Medical Research, La Jolla, CA).

polymerase forms stable complexes at this promoter even when no initiating nucleotides are present (Dobinson and Spiegelman, 1987, Whipple and Sonenshein, 1992). Protection from DNaseI digestion was also examined. Protection was tested under conditions where transcription could proceed for either 1 or 4 nucleotides. At the G2 promoter, movement of the initiation complex from 1 to 4 nucleotides downstream shifted the upstream boundary of the protected region from -24 to -18 (Figure 13). An additional band was partially deprotected at -15 when transcription had proceeded 4 nucleotides. A similar shift was not observed in protection of *abrB*, even under conditions permitting elongation of 13 nucleotides (Figure 16), bringing up the possibility that RNA polymerase:DNA interactions were different at the two different promoters. Krummel and Chamberlin (1989) found that *E. coli* RNA polymerase in an open complex protected a region from -57 to +20 on the T7 A1 promoter, and that the formation of a transcript up to 8 nucleotides long shifted the downstream edge of protection to +24 without changing the upstream protection boundaries. A region from -32 to +30 was protected by RNA polymerase on synthesis of an 11 nucleotide transcript; this change was attributed to loss of the RNA polymerase sigma factor. At this stage the transcription complex is known as the initial elongation complex. Elongation complexes protect an approximately 30 nucleotide region and have been observed with longer RNA chains (Krummel and Chamberlin, 1989). It was possible that initiation of transcription from G2 proceeded directly to an initial elongating complex, while at *abrB* the observed instability was due to the formation of a long "stressed intermediate" (discussed below), with polymerase stretched as far as possible along the DNA. Methylation protection assays and DNaseI protection of 14 nucleotide transcription complexes (as reported in Straney and Crothers, 1987) are required to substantiate this hypothesis.

The existence of two promoters at *abrB* complicates the interpretation of RNA polymerase protection of the upstream regions in that some of the protection may have been due to polymerase interactions at the P1 promoter. However, total transcription from P1 was typically less than 20% of the total transcription from P1 and P2, therefore less than 20% of the protection of DNA molecules was expected to be due to RNA polymerase bound at P1. If protection of the upstream regions were solely dependant on RNA polymerase binding to P1 while downstream protected regions were due to RNA polymerase binding to P2, the level of protection observed at upstream binding sites would be expected to have been 20% than observed in the downstream region. Differences between the level of RNA polymerase protection in the upstream and downstream sites was not observed, therefore the effects of polymerase binding at P1 were not considered to be important in the interpretations of the protection assay data.

A region on the G2 nontemplate strand from -2 to +3 was hypersensitive to hydroxyl radical attack under conditions permitting transcription of one nucleotide. When the transcript length was extended to four nucleotides, the hypersensitive region extended strongly to +6, then continued to +9. The fact that the hypersensitive sites shifted downstream the number of nucleotides that the nascent transcript was elongated by suggests that these sites represented denatured DNA at the open complex. Hydroxyl radical hypersensitive sites found at the *abrB* promoter could represent the same phenomenon as seen at the G2 promoter. These experiments did not prove that denaturation occurred. A test for denaturation would be permanganate cleavage of the proposed denatured region; permanganate specifically causes cleavage of single stranded DNA. Hydroxyl radical protection assays for the G2 template strand would also be interesting. If the pattern seen at *abrB* were

paralleled, protection of the template strand from hydroxyl radical attack would be visible in the promoter region, but hypersensitive sites may not be particularly distinctive, if they are present.

4.3.4. A model for RNA polymerase interactions with the G2 and *abrB* promoters

A model for RNA polymerase interaction with DNA at the G2 promoter is as follows. RNA polymerase wraps completely around the DNA in the region where the strands have separated. The sugar:phosphate backbone of the template strand is protected by polymerase, while the backbone of the nontemplate strand is exposed in this region. The nontemplate strand in this region may be particularly sensitive to hydroxyl radical attack because it is single stranded. Since hydroxyl radical attacks the backbone, effects which expose this region, such as decreasing the structure by denaturation, could allow increased access to the hydroxyl radical. At the G2 promoter the hypersensitive region was extended when the transcript increased in length, whereas the hypersensitive region shifted at the *abrB* promoter. The observed difference may have been due to the difference in the number of nucleotides transcribed; transcription at G2 shifted from 1 to 4 nucleotides between the two different initiating conditions while transcription from *abrB* P2 shifted from 1 to 13 nucleotides. After 13 nucleotide transcription of P2, the active site of RNA polymerase may have moved so that the region where nucleotides were exposed also moved. With a shift of only 3 nucleotides between the two lengths of transcript produced from G2 it was less likely that a major change in the exposed region of DNA would be observed. If the interpretation of the analysis of the hypersensitive sites at G2 is correct, the hypersensitive sites at *abrB* represent the position of the active site of the enzyme.

4.4. Model for RNA polymerase activity at *abrB*

Straney and Crothers (1987) proposed a model for transcription initiation that included a stressed intermediate structure created by transcription that occurs before RNA polymerase releases any upstream binding sites at the promoter. RNA polymerase was found to protect the *lacL8 UV5* promoter from DNaseI digestion from -54 to +23. Upon formation of abortive 6mer or 8mer transcripts, protection extended to +26 without RNA polymerase shifting from its stabilizing interactions with DNA upstream from the promoter. Productive transcript elongation complexes occured upon formation of an 11mer transcript, with simultaneous loss of protection of the promoter from -54 to -15. Decreased protection of the upstream region from DNaseI cleavage on formation of transcript elongation complexes has been seen in other examples as well (Straney and Crothers, 1987, Metzger et al., 1989, Krummel and Chamberlin, 1989).

The upstream boundary for RNA polymerase protection of the *abrB* promoter region remains unchanged when transcript elongation has proceeded 1 or 13 nucleotides (Figures 14 to 17). These results are similar to those obtained by Straney and Crothers (1987) for the stressed intermediate complex formed at *lacL8 UV5*, suggesting that a similar stressed intermediate complex may form at *abrB*. Protection assays carried out under conditions where elongation to 14 or more nucleotides from the P2 promoter could yield a protection pattern showing a loss of upstream binding sites typical of the elongation complex protection pattern observed by Metzger et al. (1989), however this experiment was not attempted. Protection data for open complex formation at *abrB* was not obtained when no initiating nucleotides were added, presumably because the RNA polymerase complex at this promoter were unstable without initiating nucleotides (data not shown). *B. subtilis* RNA

polymerase has been shown to transcribe from some promoters directly from unstable intermediates, without forming the stable open complex observed with *E. coli* RNA polymerase (Whipple and Sonenshein, 1992), therefore the inability of RNA polymerase to protect the *abrB* promoter region in the absence of initiating nucleotides is not unexpected.

In *E. coli, lac* repressor binding has been shown to increase RNA polymerase binding to the *lac UV5* promoter (Straney and Crothers, 1987). RNA polymerase and lac repressor were shown to bind the *lac UV5* promoter simultaneously, and the *lac* repressor prevents formation of the open complex (Straney and Crothers, 1987). Because RNA polymerase is bound to the promoter at the same time as the repressor, when the repressor binds an inducer molecule and releases the DNA, transcription initiation is able to proceed immediately. A similar mechanism of action appeared to occur at *abrB*. RNA polymerase and Spo0A were able to bind simultaneously to the *abrB* promoter (Figures 14 to 17). At low levels of Spo0A transcription of *abrB* increased (Figure 9, EcoRI/HindIII promoter fragment), suggesting that Spo0A stimulated transcription, possibly by increasing the stability of RNA polymerase bound to the *abrB* promoter. Sporulation initiation is reversible at stage zero (Errington, 1993). If a nutrient that would obviate the need to sporulate were to appear, the ability to block sporulation initiation as quickly as possible would be advantageous to the cells. The ability to quickly increase *abrB* transcription by having polymerase already bound to the *abrB* promoter would assist in this sudden response. This stimulation was not observed when the EcoRI/Asp700 fragment was used. It is possible that stimulation also occurred with the EcoRI/Asp700 fragment, but Spo0A concentrations low enough to detect the effect were not tested.

4.5. Spo0A binding to the dual 0A boxes at *abrB* may be cooperative

DNaseI protection assays showed that Spo0A and Spo0A~P bound to the *abrB* promoter over a concentration range of 100 nM protein. Binding was not evident at 300 nM Spo0A or Spo0A~P, while protection was evident at 350 nM Spo0A and Spo0A~P on the nontemplate DNA strand and at 400 nM Spo0A and 350 nM Spo0A~P on the template DNA strand (Figures 10 and 11). Protection of the *abrB* promoter region was therefore sensitive to small changes in Spo0A or Spo0A~P concentration. Further evidence of this sensitivity was seen in transcription assays testing different concentration inputs of Spo0A or Spo0A~P (Figure 9). The protein concentration at which no transcription inhibition occurred was 250 nM for Spo0A and 50% inhibition was obtained with 200 nM for Spo0A~P. There are two 0A boxes at +11 and +22 on the *abrB* promoter (Figure 1), and the presence of a second 0A box at *abrB* may be an important factor in the sensitivity of the promoter to Spo0A regulation (Hoch, 1993). Cooperative binding of Spo0A at the two 0A boxes was implicated by the narrow range in concentration over which protection of *abrB* DNA by Spo0A occurred (Figures 10 and 11). The sensitivity of *abrB* transcription may also explain the differences between Spo0A regulation seen with the *abrB* EcoRI/HindIII and EcoRI/Asp700 promoter fragments. Transcription from the EcoRI/Asp700 fragment was repressed at lower levels of Spo0A or Spo0A~P than from the EcoRI/HindIII fragment. The EcoRI/Asp700 promoter fragment does not have the upstream 0A box (Figure1), therefore it has only 2/3 the defined Spo0A binding sites that are present on the EcoRI/HindIII promoter fragment. The presence of an extra 0A box on the EcoRI/HindIII fragment could have titrated away 1/3 of the eavailable Spo0A at a given protein concentration, thus having the observed significant effect on transcription stimulation and inhibition shown in Figure 9.

4.6. Role of phosphorylation in Spo0A inhibition of *abrB* transcription

4.6.1. Differences between repression of *abrB* transcription by Spo0A and Spo0A~P

Repression of transcription from the *abrB* promoters P1 and P2 to 50% of the control level of transcription by Spo0A~P typically occurred at a two to five fold lower protein concentration than repression by Spo0A. Apporoximately 800 nM Spo0A repressed transcription of 2 nM *abrB* to 50% of the transcription level observed in the control samples, whereas 50% repression of *abrB* transcription was achieved by 150 nM Spo0A~P (Figure 9). Hydroxyl radical protection assays showed Spo0A mediated changes in RNA polymerase protection of *abrB* DNA at 400 nM protein which were evident at 100 nM Spo0A~P (Figure 17). Protection of *abrB* DNA from DNaseI cleavage in previous work showed that Spo0A~P bound the *abrB* promoter region at 20 to 50 fold lower protein concentrations than did Spo0A (Trach et al., 1991). The experiments by Trach et al. (1991) were done in 120 mM KCl, while the experiments in this thesis were conducted in 80 mM KAc. KCl has previously been shown to interfere with protein:DNA interactions (Liermo et al., 1987). The ability of Spo0A or Spo0A~P to protect *abrB* from DNaseI cleavage was therefore compared in the presence of 100 mM KCl or 100 mM KAc. The concentrations of Spo0A or Spo0A~P required to protect *abrB* DNA from DNaseI were not significantly different in the presence of either salt (Figure 12), therefore it is unknown why the results between previous work and this thesis differ significantly.

4.6.2. A model for the role of Spo0A and Spo0A~P in repression of *abrB* transcription during sporulation initiation

Spo0A accumulation in the cell is an indication that sporulation initiation has already occurred, because it requires the repression of *abrB*

transcription and activation of transcription of *spo0H* (Strauch and Hoch, 1993). During the transition state, Spo0A is produced at low levels in *B. subtilis* and sporulation remains repressed. Sporulation is initiated by activation of the phosphorelay, which in turn requires repression of *abrB* transcription. The concentration of Spo0A~P required to repress *abrB* transcription by 50% was shown in my experiments to be two to five fold less than the required concentration of Spo0A, and *abrB* transcription was sensitive to small changes in Spo0A~P concentration (50 to 100 nM protein). It would therefore be possible to repress the production of AbrB without the production of increased amounts of Spo0A. Transcription levels from *abrB* promoters P1 and P2 on the EcoRI/HindIII DNA fragment (Figure 1) decreased by 80% betwen 200 and 400 nM Spo0A~P (Figure 8). The sensitivity of the *abrB* P1 and P2 promoters to small changes in the phosphorylation state or concentration of Spo0A is probably necessary for Spo0A phosphorylation to have an effect on *abrB* transcription in vivo. The phosphorelay protein Spo0F is virtually nonexistant in cells undergoing vegetative growth (Hoch, 1993), therefore unless an alternate pathway for Spo0A phosphorylation exists, the initial *abrB* transcription repression required for sporulation initiation would not be possible.

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