The Role of Curved DNA in Promoter Selection by the Major Bacillus Subtilis RNA Polymerase.

Carl Feemster Mcallister

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The role of curved DNA in promoter selection by the major
*Bacillus subtilis* RNA polymerase

McAllister, Carl Feemster, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1988
THE ROLE OF CURVED DNA IN PROMOTER SELECTION BY THE MAJOR
BACILLUS SUBTILIS RNA POLYMERASE

A dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
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Doctor of Philosophy

in

The Department of Microbiology

and

Interdepartmental Studies in Genetics

by

Carl Feemster McAllister
B.A., B.S., University of Mississippi, 1977
M.S., University of Mississippi, 1982
August, 1988
DEDICATION

This research is dedicated to the memory of my mother Mrs. Ann Feemster McAllister, and to my wife Sigrid. I would never have progressed to the level of capability necessary to enter a doctoral program without the love and encouragement of my mother. Her courage in the face of adversity has been an inspiration to me. Her belief in me made me aspire to higher goals and gave me the perseverance to realize these goals.

My wife Sigrid's tireless efforts allowed me to concentrate my energies on the completion of this research and the writing of this dissertation. In spite of a grueling schedule at work and in our home she was always there to lift my spirits. She never let me give up on myself, even during the most discouraging periods. She and our son Cameron have given me a reason to strive onward and upward and have provided me with more happiness than I ever imagined I could have.
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I would like to express my sincere gratitude to my major professor and mentor Dr. Eric C. Achberger. His patience, encouragement and invaluable advice have greatly facilitated the completion of this research and the preparation of this dissertation. His enthusiasm and knowledge have assured that I leave LSU with a better understanding of science and life than I arrived with. I could not have chosen a more capable member of this department to help me learn molecular biology.

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ABSTRACT

The major RNA polymerases of Bacillus subtilis and Escherichia coli recognize the same conserved sequences at -10 and -35 of the promoter. The B. subtilis enzyme requires additional DNA sequences for efficient promoter utilization. Deletion mutants were used to demonstrate that the function of two B. subtilis phage SP82 promoters, Ball29 and Alu156, was dramatically affected by sequence upstream from -35. The upstream DNA contained runs of four to six adenines with a 10-11 base pair periodicity. Indicative of DNA curvature, these regions decreased electrophoretic mobility of DNA fragments containing either promoter. The curved upstream DNA increased the affinity of both promoters for RNA polymerase. This resulted in increased in vivo transcription from Alu156 but decreased transcription from Ball29. The extremely stable RNA polymerase:DNA complexes formed with the Ball29 promoter suggested that transcription from this promoter may be limited at promoter clearance following initiation. Transcription from the Alu156 promoter was limited at the level of RNA polymerase binding and strongly dependent on the curved upstream DNA. Alu156 mutant promoters were constructed by insertion of oligonucleotides between the -35 region and the curved DNA to examine the structure/function relationship of these two regions. The incremental insertions altered the rotational orientations as well as the distance between the promoter and upstream curved DNA. In vivo expression, in vitro transcription, and RNA polymerase binding were all modulated by the rotational orientation. The most efficient mutant promoters were
those in which the original orientation was preserved. The electrophoretic mobilities of these mutants suggested that the upstream curve directionally opposes a downstream curve, conferring an S shape to the DNA of this promoter. These results are consistent with a model in which the RNA polymerase either directly contacts the curved DNA resulting in additional protein-DNA interactions through a wraparound effect or contacts DNA upstream from the polyadenine tracts using a curvature-mediated DNA looping mechanism. The Ball29 and Alu156 upstream regions were used to increase in vivo expression from the lambda phage PL and PR promoters in B. subtilis. No correlation was observed between RNA polymerase binding and expression for the hybrid lambda promoters.
INTRODUCTION

Regulation of Transcriptional Initiation in Procaryotes
Regulation of Transcriptional Initiation in Procaryotes

The initiation of transcription by RNA polymerase represents the first step in the production of a product from a DNA template, or gene. The regulation of gene expression at this level is very economical for the cell. That is, energy is not expended to produce appreciable quantities of a specific RNA message until its product is needed by the cell.

There are three basic components in the control of transcription initiation: 1) RNA polymerase—the DNA dependent RNA polymerase which catalyzes the synthesis of RNA, 2) Promoters—the specific DNA nucleotide sequence at which the RNA polymerase binds and initiates transcription, and 3) Regulatory molecules—small proteins which interact with the RNA polymerase and/or the DNA resulting in the activation or repression of transcriptional initiation.

RNA polymerase. The most widely studied procaryotic RNA polymerase is that of Escherichia coli. It is a multimeric enzyme with several different subunits: beta-prime (β', molecular mass 155,613 daltons), beta (β, 150,618 daltons), alpha (α, 36,512 daltons and present as a dimer), sigma (σ, 70,263 daltons for the most common form) (McClure, 1985) and omega (ω, 10,105 daltons) (Burgess, 1976; Gentry and Burgess, 1986). These subunits aggregate to form the RNA polymerase holoenzyme. The sigma subunit is responsible for the recognition of the specific sequence of the promoter by the holoenzyme (Burgess, et al., 1969). Following initiation and the synthesis of approximately 8 bases of the transcript, sigma dissociates from the holoenzyme (Hansen and McClure, 1980), leaving
the core enzyme to elongate the transcript. Following termination, the released core RNA polymerase binds a free sigma and may then initiate another round of transcription.

After the discovery of the E. coli rpoD gene product, sigma-70, it was proposed that different sigma subunits might function in regulation by directing initiation from distinct classes of promoters (Burgess and Travers, 1970). Recently, two other sigma factors have been identified in E. coli. The heat shock response (Neidhart, et al., 1984) in E. coli is regulated by the rpoH gene product ($\sigma^{32}$, 32,381 daltons). Holoenzyme formed from the addition of core to purified $\sigma^{32}$ (i.e. E$\sigma^{32}$) initiated transcription from heat shock promoters but not from promoters recognized by E$\sigma^{70}$ (Grossman, et al., 1984).

E. coli and other enteric bacteria also contain a third sigma factor which is involved in the transcription of nitrogen regulated genes. Under nitrogen limiting conditions, the ntrA (rpoN) gene product (i.e. $\sigma^{54}$) has been shown to increase the transcription of glutamine synthetase, amino acid transport and degradation genes, nitrogenase, and related genes (Garcia, et al., 1977). The mechanism of $\sigma^{54}$ regulation of glutamine synthetase will be discussed in greater detail later.

A fourth sigma subunit known to interact with E. coli RNA polymerase core is a T4 phage gene product. Immediately after infection, early T4 genes are transcribed by the unmodified host E$\sigma^{70}$ RNA polymerase. The RNA polymerase is modified by the addition of five phage encoded polypeptides before transcription of the phage late genes. Only one of these (gp$^{55}$, 23,000 daltons) has been shown
to be essential for in vitro transcription from phage late promoters, and is thus considered to be a sigma subunit (Kassavetis, et al., 1984).

The core enzyme of Bacillus subtilis is remarkably similar in subunit composition to that of E. coli. The B. subtilis enzyme contains a beta, beta-prime, 2-alpha, sigma, omega-1, and omega-2 as well as a small (21,000 daltons) subunit known as delta (δ) (Pero, et al. 1975). The role or the delta subunit has been shown to be the discrimination of DNA sequences (Achberger and Whiteley, 1981; Whiteley, et al., 1982). It reduces non-sequence specific DNA binding and enables the holoenzyme to differentiate between strong and weak promoters. The major vegetative polymerase, Ec43, contains a sigma subunit which is smaller than the E. coli σ^70 subunit. The B. subtilis rpoD gene which encodes a sigma peptide of 43,000 daltons, shows significant DNA sequence homology to its E. coli counterpart.

More alternate sigma factors are known for B. subtilis than E. coli; the former undergoes the complex developmental process of sporulation in response to environmental conditions such as carbon, nitrogen, or phosphorus starvation. At least four minor forms of sigma subunits are present in B. subtilis vegetative cells: σ^37, σ^32, σ^30 and σ^28. Both σ^37 and σ^32 appear to regulate the transcription of genes expressed after exponential growth (Wang and Doi, 1984). Sigma-28 (Jaehning, et al., 1979) may control genes which respond to the nutritional state of the bacterium before and at the beginning of sporulation. This sigma may be the B. subtilis equivalent to E. coli σ^32, as evidenced by the fact that B. subtilis
Ecoli RNA polymerase was able to direct in vitro transcription of an E. coli heat shock promoter (Briat, et al., 1985) Sigma-28 also appears to be important in the transcription of flagellar and chemotaxis genes in B. subtilis (Helman and Chamberlin, 1987).

At least two sporulation specific sigma subunits have been identified from B. subtilis. The best characterized of these is σ29 (Haldewang, et al., 1981). It is encoded by the spoIIG gene and is only present in sporulating cells. Mutations in spoIIG arrest spore development before a detached spore within the mother cell can develop. Sigma-29 is produced first as a 27,652 daltons precursor which is processed to an active form of about 24,000 daltons. A second sporulation specific sigma subunit (σ22) may be encoded by the spoIAC gene (Port and Piggot, 1981). This gene encodes a product similar in size to an RNA polymerase core-associated protein isolated from sporulating cells.

Overlapping and/or tandem promoters have been found for almost every sporulation gene or operon characterized in Bacillus. The spoVG gene is transcribed from two overlapping promoters: one recognized by Eσ37 and one by Eσ32 (Johnson, et al., 1983). Sigma-30 (Carter and Moran, 1986) also has the capability to transcribe from the spoVC promoter. The spoVC gene can be transcribed by Eσ37, Eσ32, and Eσ29 (Tatti and Moran, 1985). These arrangements allow a more precise level of transcriptional control during different developmental stages and changes in environmental conditions.

Finally, there are several phage encoded sigma factors which alter the specificity of Bacillus subtilis core RNA polymerase production. The lytic phages SP01 and SP82 are very similar; each
requires a phage encoded sigma, gp$^{28}$ in SP01, for transcription of phage middle genes, and two more subunits, gp$^{33}$ and gp$^{34}$ in SP01, for transcription of late genes (Geiduschek and Ito, 1982). Phage φ-29 requires its own gp4 for transcription of late genes.

**Promoters.** Promoters are specific sequences of DNA found in front of genes or operons to which RNA polymerase binds and initiates transcription. A comparison of the nucleotide sequence of 128 promoters from *E. coli* was made by Hawley and McClure (1983). They proposed a consensus promoter sequence after examining the base distribution at each position on the DNA in these promoters. The -35 consensus region consists of the hexanucleotide 5' TTGACA 3' and at the -10 region, or Pribnow box, the consensus sequence was found to be 5' TATAAT 3'. A spacer region of 17 base pairs between these two regions was a conserved feature in *E. coli* promoters. These findings agreed with those of previous researchers (Rosenberg and Court, 1979; Pribnow, 1975; Siebenlist et al., 1980). Mulligan, et al. (1984) have devised a computer search program for promoter based on the *E. coli* consensus promoter sequences. An updated program with sequence realignment, was recently written and used to analyze an additional 100 promoters (Harley and Reynolds, 1987). They found all bases of the -35 and -10 regions to be highly conserved. The spacer region was 16-18 bp for 92% of the promoters, and the transcriptional start site was 6-8 bp downstream from the -10 region in 75% of these promoters.

For the major *B. subtilis* enzyme, E$^{43}$, the -35, -10 and spacer regions appear to be identical to those conserved for *E. coli* E$^{70}$ promoters (Moran, et al., 1982; Graves and Rabinowitz, 1986). The
E. coli and B. subtilis RNA polymerase holoenzymes containing the alternate sigma subunits recognize promoters with unique conserved sequences (Doi and Wang, 1986). Promoters recognized by the minor polymerases Ec37, Ec32, Ec29, and Ec28 of B. subtilis and Ec32 of E. coli all possess unique conserved sequences at -35 and -10 and display small differences in length of the spacer region. Two exceptions to this trend have been observed. The E. coli Ec54 promoters have conserved regions at -20 and -10 separated by only three nucleotides. For T4 Ecgp55, there appears to be only one conserved sequence at -10.

The conserved bases in the -35, in the spacer (e.g. the -16 region), and in -10 regions have been shown to be sites of interaction with RNA polymerase indirectly through the study of mutants and directly by using enzymatic and chemical probes. Enzymatic probes include endonucleases such as DNase I and exonucleases such as exonuclease III. Large molecules such as these are useful in identifying the outer limits of interaction of RNA polymerase with the promoter. Small chemical probes can be used to specifically identify points of interaction between the enzyme and promoter. These include dimethylsulfate (DMS) which methylates the N3 of adenine in the minor groove and N7 of guanine in the major groove of the DNA double helix, and ethylnitrosourea which ethylates phosphates on the DNA backbone. More recently, 1,10 phenanthroline-copper ions which when reacted with H2O2 nick the DNA with a preference for secondary structure (Spassky and Sigman, 1985), and hydroxyl radicals which extract a hydrogen from the deoxyribose ring to break the DNA backbone (Burkhoff and Tullius, 1987) have been
employed as chemical probes. Additionally, interactions between RNA polymerase and thymine residues of promoters have been detected by replacing thymine with 5-bromouracil, and cross-linking those nucleotides that interact with RNA polymerase to the enzyme using U.V. irradiation.

For RNA polymerase interaction studies, the DNA is first uniquely end labeled before further treatment. Two basic types of experiments have been used. In a protection experiment, RNA polymerase is allowed to bind the promoter, which is then treated with enough probe to give one modification per strand of the DNA. This type of experiment detects which nucleotide and backbone positions are protected by bound RNA polymerase due to the proximity of the enzyme. In premodification experiments, the DNA is first treated with the probe and then reacted with RNA polymerase to determine which of the modifiable sites on the promoter are necessary to form stable RNA polymerase:promoter complexes. Bound DNA is separated from unbound DNA by filtering through a nitrocellulose membrane filter followed by strand breakage, if necessary, and electrophoresis on a denaturing, polyacrylamide gel of the type used for nucleotide sequencing.

A number of studies using E. coli Eσ^70 (Siebenlist and Gilbert, 1980; Siebenlist, et al., 1980; Simpson, 1979a; Simpson, 1979b; Simpson, 1982; Dubendorff, et al., 1987) and B. subtilis Eσ^43 (LeGrice and Sonenshein, 1982; Achberger et al., 1982; Whiteley, et al., 1982) have shown that all the RNA polymerase promoter contacts are initially on the same side of the DNA helix at the -35 and -16 regions. Open promoter complexes (i.e. RNA polymerase:promoter
complexes in which the DNA strands are separated from about -9 to +3) may be distinguished from closed complexes by the increased susceptibility of cytosine residues in the melted region of the open complex to DMS attack. Although DNase I and exonuclease protection experiments have demonstrated that the promoter is protected up to -45 to -50, there has been little evidence presented using small probes to demonstrate specific interactions between RNA polymerase and nucleotides upstream from the -35 region. In one study (Siebenlist and Gilbert, 1980), an adenine residue at -41 on the bacteriophage T7 A3 promoter was shown to be protected from methylation.

For a number of years, the temporal sequence of RNA polymerase:promoter interactions during transcriptional initiation were defined by the following model (Walter, et al., 1967; Chamberlin, 1974):

\[ R + P \overset{K_B}{\leftrightarrow} R_P C \overset{K_P}{\rightarrow} R_P O \]

where R and P represent RNA polymerase and promoter, respectively, which bind reversibly to form the closed promoter complex, R_P C. A slow, thermodynamically favorable and rate limiting isomerization then occurs to give the open promoter complex, R_P O, at which transcription initiates in the presence of the necessary nucleoside triphosphates. This is followed by promoter clearance, marking the end of initiation and the beginning of elongation of the transcript. The open promoter complex represents a specifically perturbed, promoter structure that has undergone a cooperative unwinding and separation of the DNA strands. In addition to responding to the promoter sequence, the pathway kinetics are affected by temperature
and the extent of DNA template superhelicity. Recent evidence from several laboratories indicates that the addition of at least one more intermediate is necessary to explain results obtained by varying the aforementioned parameters. The amended model is as follows:

\[
\begin{align*}
    R + P & \rightleftharpoons_{K_B}^{K_F} R_P C \rightleftharpoons_{K_i}^{K_f} R_P I \rightleftharpoons_{K_I}^{K_P} R_P O
\end{align*}
\]

The evidence for this new intermediate, \( R_P I \), came primarily from the finding that after lowering the temperature, the inactivation of transcribable RNA polymerase:promoter complexes is at least 10 times faster than the rate of dissociation of binary complexes when challenged with poly \([d(AT)]\). This was deduced to be due to a rapid, reversible isomerization step \( (R_P O \rightleftharpoons R_P I) \) that must occur prior to the isomerization which yields poly\([d(AT)]\) sensitive complexes \( (R_P I \rightleftharpoons R_P C) \). The two step model would predict a decreased dissociation rate with decreasing temperature, whereas the data show an inverse relationship between these two parameters (Buc and McClure, 1985).

Temperature shift experiments were used to demonstrate that active complexes formed at 37°C initially transcribed at a higher rate (burst) when rapidly initiated at a lower temperature (e.g. 19°C). Within a few minutes, the rate declined to that observed for complexes not pre-incubated at 37°C. This phenomenon was reversible and independent of RNA polymerase concentration, and was about 60 fold faster than dissociation kinetics. Initiation by the addition of RNA polymerase to a 19°C reaction mixture showed a characteristic lag time, necessary for formation of an active complex, which was dependent on polymerase concentration. If complexes were formed at 19°C and rapidly initiated at 37°C, no burst or lag was seen even
when poly[d(AT)] was added. This suggests that the poly[d(AT)] resistant, transcriptionally inactive species formed at the lower temperature was quickly converted to an active form upon the upshift to 37°C.

Negatively supercoiled lacUV5 promoters showed no burst upon temperatures downshift from 37°C to as low as 9°C and exhibited no lag on upshift from 9°C to 37°C. This implied that the poly[d(AT)] resistant, inactive species was not present in a supercoiled template (i.e. all the complexes had progressed to the open form.) Above 25°C, the rate limiting step appeared to be the conversion of RPe to RPi. The Kp for linear and supercoiled templates were on the same order of magnitude and showed the same temperature dependence. Below 25°C, the Kp for the linear template decreased abruptly and the rate limiting step became the conversion of RPi to RP0. Temperature appeared to primarily affect Kp and Kf whereas negative supercoiling affected all three rate constants. Unwinding was postulated to occur prior to the conversion of RPi to RP0.

Essentially the same conclusions were published for the lambda Pr promoter (Roe, et al., 1984, 1985) and for the E. coli tetR promoter (Duval-Valentin and Ehrlich, 1986, 1987). Additionally, with the tetR promoter, structural changes in the RNA polymerase:promoter complexes were observed at 15°C, 20°C, and 30°C by probing with DMS. DNase I protection experiments showed the same pattern for all three temperatures. At 15°C, a heparin resistant form of the complex existed, but it was characterized by a rather slow transition to the open promoter complex. There was no difference in methylation pattern between RNA polymerase:tetR
promoter complexes formed at 15°C and that of free promoter DNA. If the complexes were formed at 20°C, some protection began to appear at the -16 region. A characteristic enhancement of methylation of guanine residues at positions -35, and -37, was faintly detectable. These complexes were rapidly converted to open complexes upon temperature upshift to 30°C. With complexes formed at 30°C, the cytosines at positions -8, -5, -4, and -2 on the antisense strand were identified as more reactive (i.e. single stranded DNA). Upon a downshift in temperature from 30°C to 15°C, methylation enhancement at G-37 is lost before protection at G-14 and G-15. Thus, the stable complexes formed at 20°C seem to have undergone some conformational shift allowing them to rapidly melt the DNA when shifted to 30°C. The change in reactivity at G-37 seems to precede open promoter complex formation and thus be associated with this conformational shift. The extra intermediate, apparently present during initiation from the tetR promoter, may be a consequence of the promoter's unusual structure. It is a weaker promoter than the lacUV5 or lambda Pr promoters; the sequence differs from the consensus -35 and -10 regions in 2 nucleotides each and these regions are separated by a 21 bp spacer region. In another study, transcriptionally active binary complexes of the trp promoter were found in which single stranded regions could not be detected (Kirkegaard, et al., 1983).

The unwinding step in transcriptional initiation has been further studied by several researchers. There are differences in these reports which are probably due to the use of different promoters and experimental conditions. It seems clear, however, that
initiation of transcription for these promoters proceeds through a stable non-initiatable intermediate in which unwinding of the promoter has occurred.

Amouyal and Buc (1987) assayed topological unwinding of the lac wild type promoter and lacUV5 promoter in relationship to protection patterns and formation of transcribable complexes. They concluded that unwinding, approximately 1.7 turns of the helix, of the weak wild type and strong lacUV5 promoters was comparable. For both promoters, it appeared that the vast majority of unwinding took place before the formation of open complexes occurred. The effect of negative supercoiling was equivalent for both the P₁ and P₂ tandem promoters on the wild type lac promoter. Negative supercoiling of the template stimulated transcription from both promoters in the absence of the cAMP receptor protein (CRP) probably due to the ease of formation of an inactive (e.g. RP₁) intermediate. Amouyal and Buc (1987) further suggested that the unwinding could be in the form of untwisting or "negative writhe". Negative writhe would be expected to bend the DNA helix, which they claim would be energetically unfavorable. However negative writhe has been detected in the bending of DNA around HU histone-like proteins (Broyles and Pettijohn, 1986).

Other researchers (Brahms, et al., 1985) investigating the effects of supercoiling on transcription from pBR322 promoters, concluded 1) that negative supercoiling increased transcription from the rep, bla, and tet promoters and 2) that this stimulation was attributable to an increase in axial writhe. They observed that this axial writhe was accompanied by deviation in the DNA structure from B
form (e.g. the formation of kinks at certain sites). Axial bending of a DNA molecule was observed at negative superhelical densities which were still less than that of natural supercoiled DNA. It was suggested 1) that this axial bending would generate an A-DNA region capable of forming a kink and 2) that kink or bend formation could lead to a local decrease in base stacking at or near the promoter. This would enhance the formation of the transcribable open promoter complex. Thus at least one effect of supercoiling appears to be increasing a rate limiting step in transcription initiation, either by untwisting the DNA or by increasing axial writhe.

**Regulatory molecules.** Transcriptional initiation is influenced by factors other than promoter sequence and RNA polymerase subunit composition. Repressors, proteins which prevent the transcription of a gene or operon, were first proposed for the regulation of the lac operon by Jacob and Monod (Jacob and Monod, 1961). Repressor proteins typically bind to a region called an operator which usually overlaps the promoter for the gene which is under negative control. For the lac repressor, this site exhibits dyad symmetry and spans the region from -2 to +23 (Schmitz and Galas, 1979). The repressor is bound in the absence of the inducer, allolactose. Allolactose acts as an allosteric effector when it binds the repressor, greatly reducing its affinity for the operator site. Thus, in the presence of inducer, RNA polymerase is not blocked by repressor and may transcribe the genes of the lac operon.

Positive regulation by ancillary proteins was demonstrated shortly after negative control was described (Englesberg, et al., 1965; Garen and Echols, 1962). The most widely studied activation
protein is the cAMP receptor protein (CRP), which is important in the positive regulation of lac, mal, ara, gal, and other operons (de Crombrugghe, et al., 1984). Other molecules such as the araC (Ogden, et al., 1980), malt (Debarbouille, et al., 1978), ompR (Hall and Silhavay, 1981), ntrC (Magasanik, 1982) and nifA (Ow and Ausubel, 1983) gene products also serve as activators for various operons. Typically, but not always, an activator, such as CRP, binds directly upstream from a promoter adjacent to or overlapping the -35 region.

It is important to note that activators can also function as repressors. The lambda cI protein bound at O\textsubscript{R2} prevents binding of P\textsubscript{R} by RNA polymerase and thus represses the cro, cII, O, P operon. At the same time, it stimulates its own transcription from P\textsubscript{RM} (Ptashne, 1978). Likewise CRP stimulates transcriptional initiation of the galP\textsubscript{1} promoter while repressing transcription of the galP\textsubscript{2} (Busby, et al., 1982). Promoters which require positive regulation by activators generally have low activity in the absence of activator due to poor homology with promoter consensus sequences.

In addition to models that propose the repressor sterically blocks the RNA polymerase from binding to the promoter, altered DNA conformation (e.g., DNA bending or looping) plays a role in repression. One of the better examples of DNA looping is observed with the repression of the gal operon (Herbert, et al., 1986; Majumdar and Adhya, 1984). The repressor binds at two distinct operators, O\textsubscript{C} and O\textsubscript{I}, located either side of the gal operon promoters. The dimerization of the DNA-bound repressors creates a loop of over-twisted DNA containing the promoters. This altered DNA conformation effectively prevents RNA polymerase binding. Another
example of the looping model is the arabinose operon (Dunn, et al., 1984; Martin, et al., 1986). The arabinose operon consists of the araC gene and its promoter and the araBAD gene cluster and their promoter. The two promoters are directionally opposed and the start sites are separated by approximately 150 bp. The AraC protein acts as a repressor of its own synthesis and the synthesis of araBAD gene products in the absence of arabinose. Results from deletion and insertion experiments suggest that repression in the absence of arabinose is most easily explained by the formation of a 210 bp loop of DNA between different AraC dimers bound at a site downstream from the araC gene and at the araBAD regulatory region upstream from the araC gene. In this way, the AraC at the araBAD site is held in the repressor conformation until arabinose becomes available and cAMP levels are elevated. The insertion of oligonucleotides between the two AraC binding sites demonstrated that the sites must be on the same side of the helix for repression to occur.

DNA bending appears to be involved in the activation of transcription by ancillary proteins such as CRP and NifA. The binding of cAMP–CRP complex at the lac promoter has been shown to bend the DNA (Wu and Crothers, 1984). This bend has been estimated to be on the order of 90-180° (Liu-Johnson, et al., 1986). Deletions or insertions of DNA between the CRP binding site and the -35 region of the lacP1 promoter virtually eliminated CRP activation. Low level stimulation was restored for insertions of 11 base pairs which would maintain the orientation of the site with respect to the promoter (Yu and Reznikoff, 1984; Mandecki and Caruthers, 1984). DNA looping has been proposed to explain NtrC stimulation of the glnABG operon.
(Reitzer and Magasanik, 1986) and NifA stimulation of nif operon transcription (Buck, et al., 1986). In each case, the activator binding sites are located far upstream (i.e. greater than 100 base pairs) of the promoter, and separation of these two regions by more than 1000 base pairs does not effect stimulation. The activator bound upstream is believed to loop the DNA around and bind the RNA polymerase:promoter complex.

Sequence-Dependent DNA Curvature

Examples of sequence-dependent DNA Curvature. In the past few years, several double stranded DNA molecules have been shown to display an altered conformation (i.e. sequence-dependent DNA curvature). The first example reported was kinetoplast minicircle DNA of trypanosomes such as Leishmania tarentolae (Challberg and Eglund, 1980; Simpson, 1979). Kinetoplast DNA consists of a few large maxicircles, which seem to function as the mitochondrial DNA of these parasitic eucaryotic organisms, and thousands of smaller minicircles (e.g. DNA circles of approximately 800 bp). The kinetoplast DNA is organized into thousands of catenated circles which form a network. There are three to five major and several minor classes of minicircle DNA based on restriction enzyme analyses (Kidane, et al., 1984; Challberg and Eglund, 1980). Their function is still unknown. They do not appear to be transcribed and they contain many stop codons within their DNA sequences, which indicates that even if transcribed they could not be efficiently translated. Various restriction fragments of kinetoplast minicircles have been shown to migrate abnormally slowly on polyacrylamide gels (Challberg and Eglund, 1980; Hagerman, 1984; Simpson, 1979; Marini, et al.,
These restriction fragments reacted as smaller molecules in electric dichroism relaxation time experiments (Marini, et al., 1982). The sequence of these fragments shows many runs of 4-8 consecutive adenines and a few runs of 4-5 thymines (Marini, et al., 1982; Kindane, et al., 1984). These regularly phased regions were proposed to comprise a systemically curved region of DNA, and that the curve might function to facilitate packaging of minicircle DNA into the kinetoplast or serve as a recognition site for DNA binding proteins.

Subsequent to the first reports of abnormal migration of kinetoplast DNA restriction fragments, several additional examples of regions of curved DNA have been described. In many of these cases, the presence of DNA curvature has been shown to be relevant to the biological function of these DNA regions. Eucaryotic nucleosomes have been localized to regions of DNA with a regularly phased distribution of purine and pyrimidine dinucleotides, especially 5' AAAT 3' (Mengeritsky and Trifanov, 1984). A computer generated projection of nucleosome sites based on regions of phased purine and pyrimidine dinucleotides correlated very well with areas of the DNA which were resistant to DNase I (Sargosti, et al., 1982) and to pause sites for Bal-31 nuclease (Scott, et al., 1984). Different patterns of histone binding to alternative sequence-dependent points along the genome may represent a mechanism for the manifestation of different stages of gene expression. In at least one instance, an experimental shift in nucleosome position resulted in a large change in mRNA synthesis (Wittig and Wittig, 1982). Other examples of curved DNA for eucaryotic systems include an SV40 origin of replication (Ryder,
et al., 1986), mouse satellite DNA (Trifanov, 1986), and a yeast autonomously replicating sequence (Snyder, et al., 1986).

Curved DNA has also been shown to influence protein binding in procaryotes. Notable examples include the origin of replication regions for phage lambda (Zahn and Blattner, 1985), phage ϕX174 (Trifanov, 1985), plasmid pR6K (Mukherjee, et al., 1985, plasmid pT181 (Koepsel and Kahn, 1986), and plasmid pBR322 (Trifanov, 1985). The attachment sites for the lysogenic phage lambda (Leong et al., 1985), as well as those of phages ϕ-80, and P22 (Ross et al., 1982; Ross and Landy, 1982) contain regions of curved DNA that are postulated to stimulate the binding of specific proteins. Curved DNA has also been detected associated with the promoters of highly expressed genes. These finding will be reviewed in a later section.

Models for sequence-dependent DNA curvature. There have been several models proposed to explain the mechanism of sequence-dependent DNA curving. Two of these models assume a B DNA-like conformation throughout. Hagerman (1984, 1985, 1986) has proposed a purine clash model based on a modification of the Dickerson-Calladine rules (Calladine, 1982). These rules predict helix deformation in response to a steric hindrance of consecutive purines on opposite DNA strands which cause compression of the minor groove and opening of the major groove. The purine clash model was not able to account for the differences seen in the electrophoretic mobility of ligated multimers of four different double stranded oligomers (Diekmann, 1986). These 10 bp molecules contained different sequences of four purines within the same DNA context. The purine clash model is not widely accepted at the present time.
The second model which assumes B-form DNA is the dinucleotide wedge model (Trifanov and Sussman, 1980; Trifanov, 1986; Ulanovsky, et al., 1986). For this model, it has been proposed that adenine dinucleotides within DNA sequence do not exist with the bases parallel but angled with respect to each other as if a wedge had been driven between them. This opening by a combination of tilt and roll causes a curvature of 5-15 degrees in the DNA with the adenine tracts on the inside of the curve. A number of naturally occurring DNA molecules that contain the adenine dinucleotide motif have been shown to display aberrant electrophoretic mobility. Other researchers, however, have been unable to demonstrate aberrant electrophoretic mobilities for synthetic molecules containing less than four consecutive adenines with a 10 bp periodicity (S. Diekman, 1986; Koo, et al., 1986).

The most widely tested model at the present time is the junction bending model of Crothers and coworkers. This group was able to localize the bend or curve center of a 423 bp kinetoplast DNA fragment to the middle of a region which contained four groups of five to six adenines separated by four to five bases by examining the electrophoretic mobility of a set of circularly permuted constructions (Wu and Crothers, 1984). It was also demonstrated that electrophoresing the DNAs in the polyacrylamide gels at temperatures greater than 60°C or treatment of the DNA samples with the oligopeptide antibiotic distamycin, which binds the minor groove of B-form DNA, greatly reduced these electrophoretic anomalies. The authors suggest that the adenine tracts of kinetoplast DNA exist in a non-B form, such as heteronomous, or H-DNA. The existence of H-DNA
in poly d(A)*poly d(T) regions of DNA was first proposed by Arnott et al. (1983). Further evidence from Raman spectra (Jolles et al., 1985) and linear dichroism (Edmondson and Johnson, 1985) have also suggested that poly d(A)*poly d(T) DNA exists in a non-B form, though not necessarily as H-DNA. Wu and Crothers further suggested that in both naturally curved kinetoplast DNA and in CRP induced lac promoter bending, the conformation is due to kinks or sharp bends at the junctions of B-form DNA and H-form DNA, rather than a smooth bend throughout these sequences on the DNA. Similar junction bends were modeled by Selsing, et al. (1979) to explain X-ray diffraction patterns for poly d(A)*poly d(T) DNA.

More recently, it was shown (Koo, et al., 1986) that runs of five adenines resulted in maximum distortion of electrophoretic migration when separated by five non-adenine bases. This phasing would be calculated to produce a helix screw of 10.3 bp per turn based on determined values of 10.5 for B-DNA and 10.1 for poly d(A)*poly d(T) in solution (Strauss, et al., 1981). Interruptions of adenine runs with even one base dramatically reduced curvature. It should be noted that the non-curvature of the adenine run interrupted with another purine, guanine, was not predicted by the purine clash model. When various 10 bp combinations of adenine runs and spacer sequences were assessed for anomalous electrophoretic mobility, the most curved sequence was A_5N_4 followed by A_8N_2, A_5N_5, A_9N_1, A_4N_6, and lastly A_3N_7. Since A_8N_2 and A_9N_1 appeared significantly curved, it is believed that the 3' and 5' ends of the adenine tracts do not equally contribute to curvature. As part of an elegant experiment, two sequences were constructed with the 3' end or the 5' end of the
adenine tracts phased at 10 bp intervals; the 3' junction was shown to be more important to DNA curvature. The bases flanking the adenine tracts were shown to have a lesser effect on curvature, with cytosine and thymine preferred at the 5' and 3' junction ends, respectively. Lower temperature was observed to increase the aberrant electrophoretic mobility for virtually all of the constructions except AgN₁.

The dinucleotide wedge model fails to explain the differences in curvature for the AgN₁ molecule which behaves as a curved DNA molecule and the A₃N₇ and interrupted A₂NA₂ molecules which appear slightly curved. Adenine dinucleotides (ApA) at 5 bp intervals have been shown to cancel each other. Thus, the AgN₁ molecule in which all but two of the ApA steps are canceled should be bent to nearly the same extent as the A₃N₇ and A₂NA₂ molecules which would have two dinucleotide wedges also. Based on x-ray diffraction (Arnott, et al., 1983) and optical data (Edmondson and Johnson, 1985) an unusual feature of H-DNA appears to be a nonequivalence of the two strands. The thymine residues on one strand appear to have a greater angle relative to the helix than the adenines on the opposite strand in poly d(A)·poly d(T) DNA. It should not be surprising that the tilt induced curve at the 3' junction would be larger than that at the 5' junction of an adenine run.

New data has been reported, which has led to the modification of the junction bend model. The hydroxyl radical, 'OH, was used to probe for structural features causing bending of kinetoplast DNA (Burkhoff and Tullius, 1987). This free radical nicks the strand of DNA by extracting a hydrogen atom from the deoxyribose. The
frequency of cutting at each position of the backbone is measured using uniquely end-labeled fragments run on a sequencing gel. A plot of the cutting frequency of each kinetoplast DNA strand resembled a sine curve, such that cutting frequency in the adenine tracts decreased smoothly from 5' to 3' reaching a minimum at the 3' end and smoothly increasing back to a maximum 4-5 bases further downstream from the adenine tract. The trend was just the opposite for the complimentary thymine tract on the opposite strand. These results could most easily be explained by a progressive narrowing of the minor groove from 5' to 3' for each adenine tract. The adenine tract DNA would then bend toward the minor groove at its center. This could more fully explain the effects of distamycin and netropsin, which bind the minor groove in adenine-thymine (A + T) rich sequences. These drugs would serve to open up the minor groove, causing the DNA to bend back in the opposite direction from the adenine tract-induced bend, and eliminating the curvature of the DNA.

The junction bend model would have predicted a square wave due to uniform conformation for DNA within the adenine tract and normal B-DNA flanking these regions. The junction bend model has been modified (Koo and Crothers, 1988) to include a roll component at the 5' end of adenine tracts which would open the minor groove. This puts the model in agreement with the observed hydroxyl radical cutting pattern. These modifications make the junction bend model more similar to the adenine dinucleotide wedge model.

Curved DNA associated with promoters from E. coli. The presence of A + T rich regions upstream from the -35 region of bacterial promoters, was described several years ago (Vollenweider et
al., 1979) and now seems to be emerging as a general pattern among efficiently utilized promoters (Nakamura and Inouye, 1979; Horn and Wells, 1981a, 1981b), especially for tRNA genes (Lamond and Travers, 1983; Bossi and Smith, 1983, 1984; Nishi and Itoh, 1986) and rRNA genes (Brosius, et al., 1981). These upstream A + T regions have now been implicated in the structure (i.e. DNA curvature) (Bossi and Smith, 1984; Gourse, et al., 1986; Mizuno, 1987) and function (i.e. enhanced transcription) (Bossi and Smith, 1984; Gourse, et al., 1986; Mizuno, 1987; Nishi and Ito, 1986; Travers, et al., 1983; Mizuno and Mizushima, 1986; Inokuchi, et al., 1984) of several promoters. The adenine tracts vary in number, length, and distance from the -35 region for some promoters. Using a computer program designed to detect sequence similarities, Galas, et al. (1985) analyzed 59 promoter sequences compiled by Hawley and McClure (1983). They noted the presence of an adenine tract around -44 conserved in 36 of these promoters.

With the tyrT promoter, there appear to be secondary binding sites for RNA polymerase upstream from the primary site (Lamond and Travers, 1983). These sites have lower homology to the consensus -35 and -10 sequences than does the primary site and they appear to be located within an extended A + T rich region. DNase I protection experiments demonstrated that additional RNA polymerase molecule(s) are able to bind to this upstream region (Travers et al., 1983). Deletion of this region was accompanied by decreased transcription from the tyrT promoter and loss of DNase I upstream protection. In this instance, it is unclear to what extent the observed effects are due to secondary RNA polymerase binding sites or to the curved
upstream DNA. The binding and transcription experiments were performed at low salt concentrations at which *E. coli* RNA polymerase exhibits pronounced non-specific binding, so it is questionable whether the binding upstream by additional RNA polymerase molecules is physiologically relevant. Travers (1984) has more recently attempted to show secondary binding sites on a number of coordinately regulated *E. coli* rRNA and tRNA promoters. These putative -35 and -10 regions generally have low homology to the *E. coli* consensus sequences and in about half the cases are oriented in the opposite direction from the promoter.

Recently, *E. coli* promoters were analyzed for curved upstream sequence using a weighted algorithm (Plaskon and Wartell, 1987). The results indicated that those transcribed efficiently in vivo, such as the *rrn* P1 promoters (Pettijohn, et al., 1970), the *lpp* promoter, the *his* promoter (Verde, et al., 1981), the *tyrT* promoter (Lamond and Travers, 1983), the spot 42 RNA promoter (Sagahan and Dahlberg, 1979), and the *Salmonella hisR* promoter (Bossi, 1983; Bossi and Smith, 1984) received a high curvature score. Several of the promoters that were rated as highly curved, the *rrnB* P1 (Gourse, et al., 1986), *tyrT* (Travers, et al., 1983), *Salmonella hisR* (Bossi and Smith, 1984) and *B. subtilis spoVG* promoters (Banner, et al., 1983) have all been shown by deletion analyses to be transcriptionally dependent on upstream sequence.

Curved DNA associated with promoters from *B. subtilis*. The A + T rich DNA sequence upstream from the -35 region appears to be even more conserved among *B. subtilis* promoters than for those of *E. coli*. Although *E. coli* E*0*70 and *B. subtilis* E*0*43 RNA polymerase appear to
recognize the same consensus regions at -35 and -10 (Lee, et al., 1980b; Moran, et al., 1982; Murray and Rabinowitz, 1982; Graves and Rabinowitz, 1986), several studies have shown that E. coli or coliphage promoters are not efficiently utilized by the major B. subtilis RNA polymerase (Lee, et al., 1980; Shorenstein and Losick, 1973; Wiggs, et al., 1979; Achberger and Whiteley, 1981). Thus, the B. subtilis holoenzyme seems to require additional conserved sequences for efficient utilization of a promoter.

One of these regions of additional sequence appears to be upstream from the -35 region where A + T rich regions have been noted for several strong promoters recognized by E. coli including the veg promoter (Moran, et al., 1982), several tRNA promoters (Wawrousek, et al., 1984), two phage SPO1 promoters (Lee and Pero, 1981), and several promoters from phages ø-29 and SP82 (Murray and Rabinowitz, 1982; D. Pawlyk, 1986; McAllister and Achberger, 1988). For the spoVG promoter, recognized by B. subtilis E 37 and E 32 RNA polymerases, deletion of the upstream A + T rich regions resulted in reduced in vitro transcription. Until the work presented in this dissertation, however, there had been no systematic analysis of the effect of this upstream DNA on promoter utilization by B. subtilis Eco436 RNA polymerase.

The B. subtilis veg promoter has been shown to have tandem E 43 binding sites. Transcription from the upstream binding site in the wild type promoter has not been demonstrated in vitro (Legrice and Sonenshein, 1982), however, deletion of DNA sequence containing the upstream binding site was associated with decreased transcription. At this point, it is unclear whether the generally A + T rich
upstream DNA confers curvature to the veg promoter. It is therefore not known whether the stimulation of transcription at the downstream promoter is due to the upstream binding site serving as a "loading dock" for additional molecules of RNA polymerase, or is due to some effect by a possible region of curvature adjacent to the downstream RNA polymerase binding site. Changes in DNase I protection and methylation patterns were seen up to -100. The RNA polymerase used contained no delta subunit, so it is questionable whether this binding of an additional RNA polymerase molecule is a true physiological effect.

Peschke, et al. (1985) recently reported that several E. coli promoters could be efficiently utilized in vivo and in vitro by B. subtilis E. coli RNA polymerase. In general, the E. coli promoters that were utilized contained an A + T rich region between -35 and -50.

There is very little known as to how the curved DNA upstream of the -35 region stimulates transcription from promoters in B. subtilis. The studies that follow were undertaken to define the role of curved DNA upstream from the -35 region in promoter utilization by B. subtilis E. coli RNA polymerase.
CHAPTER I

Effect of Polyadenine-containing Curved DNA on Promoter Utilization in *Bacillus subtilis*
SUMMARY

The effect of DNA upstream of the -35 region on promoter function was examined using two promoters isolated from the Bacillus subtilis bacteriophage SP82. The affinity of RNA polymerase for the two promoters in vitro differed significantly. For each promoter, the nucleotide sequence of the upstream DNA was characterized by the presence of successive runs of adenines with a 10-11 base pair periodicity. DNA fragments with the polyadenine-containing upstream DNA displayed aberrant electrophoretic mobilities when analyzed on polyacrylamide gels indicative of curved DNA. A series of mutant promoters in which the upstream DNA was deleted or altered was constructed. The curved DNA upstream of the -35 region was required for efficient RNA polymerase binding. Decreased in vitro transcription observed when the upstream DNA was deleted could be partially restored if the template was negatively supercoiled. Measurements of chloramphenicol acetyltransferase specific activity from B. subtilis strains carrying transcriptional fusions indicate that the curved upstream DNA stimulated transcription from the promoter with the weaker affinity for RNA polymerase. The curved DNA reduced the in vivo activity of the promoter with the strong affinity for RNA polymerase. One function of the curved upstream DNA may be to provide RNA polymerase-promoter interactions that facilitate open complex formation.
INTRODUCTION

The major vegetative RNA polymerase from *Bacillus subtilis*, the 43 containing enzyme, recognizes promoters that have a consensus nucleotide sequence very similar to that derived from promoters of *Escherichia coli* (Graves and Rabinowitz, 1986; Hawley and McClure, 1983; Moran, et al., 1982; Peschke, et al., 1985). In addition to the regions of conserved nucleotide sequence at -10 and -35, promoters efficiently utilized in *B. subtilis* possess conserved nucleotide sequence at -16 and -43 (Graves and Rabinowitz, 1986; Peschke, et al., 1985). The sequence centered around -43 is characterized by a short run of adenines.

DNA upstream of the -35 region has been demonstrated to be necessary for a high rate of transcription from some promoters. These promoters include the lambda P_L promoter (Horn and Wells, 1981), the *E. coli* rrnB P_l promoter (Gourse, et al., 1986), and the promoters for the *Salmonella typhimurium* hisR (Bossi and Smith, 1984) and *E. coli* tyrT (Lamond and Travers, 1983) genes. DNA fragments containing two of these promoters, the hisR gene and rrnB P_l promoters, display abnormal electrophoretic mobilities often associated with regions of curved DNA (Diekman, 1986; Hagerman, 1985; Koo and Crothers, 1987; Trifonov, 1985). For these two *E. coli* promoters, mutations in the upstream regions that reduced promoter function imparted normal electrophoretic mobility to the DNA fragments containing these promoters (Gourse, et al., 1986; Bossi and Smith, 1984). The correlation between promoters displaying a high rate of transcription initiation and regions of altered DNA conformation has also been made using nucleotide sequence analyses (Galas, et al., 1985; Plaskon and Wartell, 1987).
DNA conformation has also been implicated in the activation of transcription by ancillary proteins. The cAMP-CRP (cAMP receptor protein) complex appears to induce a bend in the DNA at -66 relative to the lactose operon promoter P1 as part of the activation process (Wu and Crothers, 1984). Protein mediated bending or looping of the DNA upstream of promoters has also been proposed for the activation of the E. coli glnAp2 promoter by the glnG (ntrC) gene product (Reitzer and Magasanik, 1986) and the activation of the Klebsiella pneumoniae nifH promoter by the nifA gene product (Buck, et al., 1986). It may be that this type of DNA bending is functionally analogous to a sequence dependent curve in the DNA upstream of a promoter.

The DNA upstream of promoters in B. subtilis can influence transcription. The B. subtilis spoVCG promoter is recognized by minor forms of the RNA polymerase, the $\sigma^37$ and $\sigma^32$ containing enzymes (Banner, et al., 1983). The utilization of the spoVCG promoter by each of these forms of the RNA polymerase was dependent on the upstream DNA.

In this report, we examine the importance of DNA upstream of the -35 region to the function of two bacteriophage SP82 promoters recognized by the major B. subtilis RNA polymerase. A series of mutants were constructed from these two promoters in which the upstream DNA was either deleted or replaced with other DNA. Using these mutant promoters, the influence of the upstream DNA on the binding of RNA polymerase and subsequent transcription was examined. The upstream DNA enhanced the binding of RNA polymerase to the downstream promoter sequences and affected the level of transcription both in vitro and in vivo. The electrophoretic analysis of DNA fragments containing these promoters was consistent with the existence of a region of curved DNA immediately upstream of the -35 region.
EXPERIMENTAL PROCEDURE

Materials. Restriction enzymes, DNA modifying enzymes, and Ribonucleases U2 and T1 were purchased from Bethesda Research Laboratories and New England Biolabs, Inc. Dideoxy- and deoxynucleotides were obtained from Pharmacia Inc. $^{35}$SDeoxyadenosine 5'-($\alpha$-thio) triphosphate, $[5,6-{^3}\text{H}]$Uridine 5'-triphosphate, and $[\gamma-^{32}]$Adenosine 5'-triphosphate were purchased from New England Nuclear. Nucleoside triphosphates were purchased from Sigma Chemical Co. All other chemicals were of the highest quality available.

Promoter isolation. DNA restriction fragments of phage SP82 DNA containing the Ball29 and Alu156 promoters were previously identified by a combination of B. subtilis RNA polymerase binding studies and hybridization studies with $^{32}$P-labeled RNA isolated 2.5 minutes after the infection of B. subtilis 168 with bacteriophage SP82 (Achberger and Whiteley, 1980). An 156 bp AluI generated DNA fragment carrying the Alu156 promoter and a 374 bp Rsal generated DNA fragment carrying the Ball29 promoter (see below for nomenclature) were each ligated to HincII digested M13mp7 RF (replicative form) to form recombinant phage. DNA sequence determination of the promoter-containing DNA inserts of these phage was accomplished by a combination of the dideoxynucleotide chain termination method (Sanger, et al., 1977) and the chemical method (Maxam and Gilbert, 1980). The nucleotide sequence for the region of the SP82 genome containing the Ball29 promoter has been reported as part of another study (Panganiban and Whiteley, 1983). The Alu156 promoter DNA fragment lacked a recognizable translational start signal, but the Rsal DNA fragment containing the Ball29 promoter included a ribosome binding site and a start codon. This ribosome binding site was deleted from the 374 bp Rsal DNA fragment by a combination of restriction enzyme and nuclease BAL-31 digestions. The resulting DNA was inserted into the HincII site of M13mp7 RF, and the
extent of the deletion was determined by DNA sequence analysis. This DNA fragment was 129 bp in length and was named Ball29. The transcription start sites of the Alul56 and Ball29 promoters were confirmed by determination of the sequence of purines in transcripts labeled with $^{32}$P at the 5'end. The conditions for in vitro synthesis of RNA in the presence of $[^\gamma-^{32}$P]ATP (Achberger, et al., 1982) and sequence determination by the method of Donis-Keller et al. (Donis-Keller, et al., 1977) have been described.

Construction of mutant promoters. DNA sequence analysis of the Ball29 and Alul56 promoters revealed HincII sites at identical positions in the conserved promoter sequence (i.e. HincII would cleave between positions -33 and -34). This unique HincII site within each of the promoters permitted the construction of series of mutants. In each of the recombinant M13 phage, the DNA containing the Ball29 or Alul56 promoter is flanked by an upstream EcoRI site and a downstream BamHI site. The promoter mutants, which will be referred to as the "hybrid promoters," were formed by the ligation of the EcoRI-HincII DNA fragment of one promoter to the HincII-BamHI DNA fragment of the other. As a result of this exchange of upstream DNA between Alul56 and Ball29, the hybrid promoters, the Alul56 hybrid and Ball29 hybrid promoters, differ from their respective original promoters in sequence upstream from -44 (Fig. 1).

In addition, three sets of mutants were constructed in which DNA upstream from the -35 region of the promoter was deleted. (i) The "-34 deletion" was produced by the ligation of promoter DNA downstream of the HincII site, the HincII-BamHI DNA fragment, to M13mp8 RF digested with SmaI and BamHI. The thymine-adenine base pairs at positions -34 and -35 were replaced by cytosine-guanine base pairs altering the sequence of the -35 region in each construction. The mutant promoters were designated the Ball29 -34 deletion and the Alul56 -34 deletion (Fig. 1). (ii) The DNA downstream of the HincII site
for the original promoters was also ligated with M13mp9 RF digested with BamHI and EcoRI in which the ends produced by EcoRI were made flush using DNA polymerase I, Klenow fragment with dATP and TTP (Maniatis, et al., 1982). The resulting recombinant RF was purified and digested with BamHI and HaeIII, and the promoter-containing DNA fragment was purified. This DNA fragment was inserted between the SmaI and BamHI sites of M13mp8 RF. These constructions, referred to as the "-36 deletions", possess the original promoter sequence downstream of -36 including an intact -35 region (Fig. 1). (iii) A 91 bp Sau3AI generated fragment of pBR322 DNA was ligated to BamHI digested M13mp8 RF and subsequently purified as a 101 bp fragment with EcoRI and BamHI produced ends. This DNA fragment was inserted upstream from the EcoRI site in the -36 deletion mutants. These promoter constructions, named Bal129 extended and Alul56 extended, lack the DNA found upstream from the -35 region in the original promoter. DNA fragments produced by BamHI digestion of these recombinant phage retain the 101 bp fragment of non-promoter DNA. The promoter containing DNA fragments from each of the above construction was ligated into the plasmid vector, pUC8.

In vivo chloramphenicol acetyltransferase (CAT) expression. For each of the promoter constructions described above, the promoter containing DNA fragment was excised from the recombinant M13mp8 RF with EcoRI and PstI. The purified DNA fragments were ligated into plasmid pPL703 (Duvall, et al., 1983) digested with the same restriction enzymes to form a transcriptional fusion with the chloramphenicol acetyltransferase, CAT, gene contained on this plasmid. These recombinant plasmids were transformed into B. subtilis LAS10 (Bacillus Genetic Stock Center, The Ohio State University; Ostroff and Pene, 1984) protoplasts using the method of Chang and Cohen (1979). Recovery of transformants was significantly increased by using 4 ml of the regeneration
medium as an overlay. Routinely, colonies of transformants with promoter-containing pPL703 were observed 3-7 days after plating on regeneration medium containing 10 µg/ml chloramphenicol and 100 µg/ml neomycin sulfate. After confirming the presence of the correct promoter DNA fragments in pPL703, the CAT specific activity of three independent clones was assayed. Transformants grown in LB broth (Maniatis, et al., 1982) were harvested four hours after the break from the exponential growth stage. Cell-free extracts were prepared by the sonic disruption of concentrated cell suspensions in 50 mM Tris-HCl (pH 7.8) plus 4 mM phenylmethylsulfonyl fluoride followed by centrifugation in a microcentrifuge (Brinkmann Instruments, Inc.) for 1 minute. CAT activity was assayed by the method of Shaw (Shaw, 1975). Protein concentration was determined as described by Bradford (Bradford, 1976).

RNA polymerase isolation. To obtain B. subtilis RNA polymerase with a high subunit content, a core-σ fraction was isolated (Achberger and Whiteley, 1981; Spiegelman, et al., 1978) and reconstituted to holoenzyme form with the addition of purified δ subunit. The δ subunit was purified from a core-δ RNA polymerase fraction by dissociating the enzyme in 6 M urea, 25 mM NaCl, 10 mM Tris-HCl, 2 mM EDTA (pH 7.8) and passage over a phosphocellulose (Whatman) column equilibrated with the same buffer. The protein fraction not retained on the column contained the δ and α subunits of the RNA polymerase. The δ subunit fraction was dialyzed against 15% glycerol, 100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA (pH 7.8) to remove urea, and the δ subunit was separated from the α subunit by DEAE-Sephadex A-25 (Pharmacia Inc.) chromatography (Spiegelman, et al., 1978). The amount of the δ subunit preparations required to completely reconstitute core-σ to holoenzyme was established by determining the concentration of the δ subunit that gave maximum inhibition of transcription from a heterologous DNA template, such as E. coli phage T7 DNA (Achberger and
Whiteley, 1981). Under the same conditions, the addition of the same quantity of the 6 subunit had a slight stimulatory affect on transcription from SP82 DNA.

Transcription assay. RNA polymerase activity with different DNA templates was measured by the incorporation of [3H]UTP into trichloroacetic acid-precipitable material (Spiegelman, et al., 1978). The transcription reaction buffer was 50 mM NaCl, 40 mM Tris-HCl (pH 7.9), and 10 mM MgCl\(_2\). In each 0.25 ml reaction, 4.5 µg of the DNA template, pUC8 DNA or derivatives of pUC8 containing the promoter constructions described above, was added. In separate experiments, the plasmid templates were transcribed in the supercoiled, covalently closed circular form and the Scal digested, relaxed form.

RNA polymerase binding assays. The B. subtilis RNA polymerase dependent retention of promoter-containing DNA fragments on a nitrocellulose membrane filter (BA85 membrane, Schleicher and Schuell) was based on the method of Jones et al. (Jones, et al., 1977). Unless otherwise stated, RNA polymerase-promoter DNA complexes were allowed to form for 10 minutes at 37°C in a buffer composed of 50 mM NaCl, 40 mM Tris-HCl (pH 7.9 at room temperature), 10 mM MgCl\(_2\). The RNA polymerase-DNA complexes were collected on the nitrocellulose membrane by filtration at a rate of 4 ml/minute and washed with 1 ml of the binding reaction buffer. DNA was eluted from the filter with gel elution buffer (Maxam and Gilbert, 1980) at 50°C and then ethanol precipitated twice. The promoter containing DNA fragments were separated by polyacrylamide gel electrophoresis, stained with ethidium bromide, and photographed with Polaroid type 55 film. RNA polymerase dependent filter retention of DNA fragments was quantitated from the photographic negatives using a BioRad video densitometer.
The competition binding assay measured the filter retention of a series of different promoter-containing DNA fragments as a function of the RNA polymerase concentration. The DNA fragments were excised from the recombinant pUC8 plasmids described above by digestion with EcoRI and PstI. The Alul56 extended and Ball29 extended DNA fragments were removed from the pUC8 vector by digestion with BamHI. In each 0.4 ml assay, 1.5 μg of digested plasmid was added for each promoter tested. The pUC8 vector DNA contains no promoters that are efficiently bound or transcribed by the B. subtilis holoenzyme (i.e. core-β43δ) and was included in the binding reaction to minimize non-specific binding, such as end binding (Melancon, et al., 1983), to the test fragments.

The rate of dissociation of RNA polymerase from preformed RNA polymerase-promoter complexes was measured, as follows. For each of the promoter constructs tested, 1.5 μg of digested plasmid was added per reaction. The complexes were allowed to form under the conditions described above except 15 μg of RNA polymerase was employed for every 1.5 μg of digested plasmid. After allowing for RNA polymerase binding, 40 μg of single stranded M13 DNA was added for each 1.5 μg of plasmid, and the reactions were filtered at timed intervals. Background levels of binding were established by the addition of RNA polymerase to a mixture of the single stranded M13 DNA and the promoter-containing DNA fragments. Initial binding was determined in the absence of added M13 DNA. Samples were processed and RNA polymerase binding quantitated as described above.

Polyacrylamide gel electrophoresis. Electrophoresis used to separate small promoter containing DNA fragments employed an 8% (60:1 monomer to bis ratio) polyacrylamide gel with a Tris-borate-EDTA (TBE) buffer system (89 mM
Tris, 89 mM boric acid, and 2.5 mM EDTA, pH 8.3). Electrophoresis was at 10 volts/cm for 4 hours. Linear plasmid molecules were electrophoresed through 4% polyacrylamide gel for 9-14 hours under similar conditions.

**General Techniques.** Plasmid DNA and M13 RF was isolated using the alkaline lysis method (Birnboim and Doly, 1979) and CsCl gradient separation in the presence of ethidium bromide. Conditions recommended by the manufacturer were used for restriction enzymes and DNA modifying enzymes. Following electrophoresis and staining with ethidium bromide, restriction enzyme generated DNA fragments to be purified were excised from polyacrylamide gels with a razor, minced, and eluted overnight at 37°C in TBE buffer. The DNA was concentrated and further purified using DE52 (Whatman) chromatography and ethanol precipitation. Transfection of *E. coli* JM101 and transformation of *E. coli* JMB3 was accomplished with CaCl$_2$ treated cells (Maniatis, *et al.*, 1982).
RESULTS

To study how transcription initiation by the major RNA polymerase from *Bacillus subtilis* is influenced by the DNA upstream from the enzyme binding site, a series of mutant promoters were constructed in vitro. The nucleotide sequence of the two *B. subtilis* bacteriophage SP82 promoters used to make the mutant promoters is presented in Fig. 2. These promoters are recognized by the major *B. subtilis* RNA polymerase, the σ^43^ containing enzyme. This RNA polymerase recognizes the same consensus sequence as the major *Escherichia coli* RNA polymerase. When the promoters used in this study were compared to the consensus sequence for *E. coli* promoters using the parameters of Mulligan et al. (1984), the Alul56 promoter was 83% homologous and the Ball29 promoter was 90% homologous. For each promoter, the nucleotide sequence upstream from the -35 region contains runs of adenines with a 10-11 base pair (bp) periodicity. In addition, the Alul56 and Ball29 promoters have identical sequence from -31 through -44. Within this region, each promoter has a unique HincII restriction enzyme site that was used to construct three classes of mutants. Hybrid promoters were formed by exchanging the DNA upstream from the HincII site between Alul56 and Ball29. The nucleotide sequences of the resulting promoters, Alul56 hybrid and Ball29 hybrid, were altered from the original promoters upstream from -44.

Promoters grouped in the second class of mutants each possessed a deletion of all promoter DNA upstream from -33 and were referred to as the -34 deletions. In these mutants, the thymine-adenine base pairs at -34 and -35 were replaced by cytosine-guanine base pairs from the vector DNA resulting in an alteration of the -35 region. The next class of mutants were constructed in such a way as to remove all promoter DNA upstream from -35. These mutants, designated the -36 deletions, retained the same -35 region as the original
promoters. A variation on the -36 deletion series of mutants were the Alul56 extended and Ball29 extended promoters in which an 91 base pair DNA fragment, chosen for its lack of promoter related DNA, was added to the upstream terminus of the -36 deletion mutant. All mutants within a series (i.e. the Alul56 series or the Ball29 series) have the same DNA sequence as the original promoters from -33 through the DNA downstream of the transcriptional start site (Fig. 1).

In vivo expression from promoter constructions.

The promoter cloning vector pPL703 (Duvall, et al., 1983) was used to establish transcriptional fusions between each of the promoters and the chloramphenicol acetyltransferase (CAT) gene carried on the plasmid vector. The levels of CAT specific activity measured for the different promoter constructions in B. subtilis LA510 are presented in Table I. The promoter Alul56 yielded the greatest CAT specific activity. Replacing the Alul56 upstream DNA sequence with that of Ball29 (i.e. the Alul56 hybrid) reduced the CAT specific activity by one-third. Deletion of the upstream DNA from the Alul56 promoter greatly decreased activity. Alul56 -36 deletion had less than 10% of the original promoters activity and Alul56 -34 deletion with the altered -35 region had only 4% of the original activity. This data is consistent with the idea that the upstream DNA is required for efficient utilization of Alul56 in B. subtilis and that the upstream region from Alul56 resulted in greater CAT gene expression than the same region from Ball29.

The Ball29 series of promoters inserted into pPL703 yielded much different results. The original Ball29 promoter allowed approximately one-half the CAT specific activity as the Alul56 promoter. The Ball29 hybrid, which has the functional Alul56 upstream DNA sequence, displayed only 38% of the CAT specific activity of the original Ball29 promoter. Deletion of all promoter
DNA upstream from -35 from Ball29 (i.e. the Ball29 -36 deletion) resulted in increased CAT specific activity. The Ball29 -34 deletion retained 92% of the activity of the original Ball29 promoter despite containing an altered -35 region as well as the deletion of upstream DNA sequences. The deletion of upstream DNA sequences had an opposite effect on the Ball29 and Alul56 promoters. To resolve the apparent discrepancy, the in vitro interaction of RNA polymerase with the promoter constructs was examined.

RNA polymerase-promoter interactions in vitro.

To test if the low in vivo promoter activity of Ball29 and Ball29 hybrid relative to the Alu156 promoter reflects weak interactions with the RNA polymerase, the binding of B. subtilis RNA polymerase to the different promoter constructs was examined. In the initial series of experiments, equal molar amounts of the different promoter containing DNA fragments competed for sub-saturating levels of RNA polymerase. Representative results of the competition binding among Ball29, Ball29 hybrid, Alu156, and Alu156 hybrid are presented in Fig. 3. Despite the similarities in overall promoter sequence, the RNA polymerase had a greater affinity for the Ball29 promoters than the Alu156 promoters. No significant binding to the Alu156 promoters was observed until the Ball29 promoters were nearly saturated by RNA polymerase. The low in vivo activity of Ball29 and Ball29 hybrid does not appear to be due to weak binding of RNA polymerase. In addition, when the binding of RNA polymerase was measured as a function of NaCl concentration in the reaction, the Ball29 promoters were bound more efficiently at higher salt concentrations (i.e. 50% of the maximal binding observed at 0.05 M NaCl was still present at 0.25 M NaCl for the Ball29 promoters while binding to the Alu156 promoters dropped to the 50% level at 0.11 M NaCl).
The in vitro competition binding assay described in the Experimental Procedures was used to compare the affinity of RNA polymerase for the promoter constructs bearing deletions with the original and hybrid promoters. In each case, DNA fragments containing promoter constructs with the intact upstream DNA region were more efficiently bound by the RNA polymerase than those in which the upstream DNA region had been deleted (Fig. 4). In examining the binding of RNA polymerase to promoter DNA fragments, no appreciable binding to promoters lacking upstream DNA regions was apparent until after promoters with intact upstream regions were nearly saturated by enzyme. The additional DNA on the Ball29 extended promoter DNA fragment marginally improved RNA polymerase binding over that observed for the Ball29 -36 deletion promoter used to construct it (Fig 4A).

The Alul56 promoter DNA fragments bound RNA polymerase more efficiently than those with the Alul56 hybrid promoter sequence (Fig. 4B). RNA polymerase binding to DNA fragments bearing the Alul56 deletion mutant promoters paralleled that observed for the Ball29 series. Even at relatively high concentrations of RNA polymerase, DNA fragments with promoter sequences deleted of upstream regions were inefficiently bound by enzyme.

The rate of dissociation of RNA polymerase from each of the promoter constructs was measured to generate an estimate of RNA polymerase-promoter complex stability. The half-life for RNA polymerase dissociation from Ball29 hybrid promoter containing DNA fragments was consistently longer than that for Ball29 (Table II). The removal of upstream DNA from the Ball29 promoter resulted in a greater than 10 fold reduction in RNA polymerase-promoter complex stability. RNA polymerase dissociated from complexes with the Alul56 series of promoters with half-lives on the order of 1 minute. Estimates of RNA polymerase binding and RNA polymerase-promoter complex stability for the Ball29
and Alu156 promoters suggest that the RNA polymerase binds the Ball29 promoters more tightly than the Alu156 promoters. In general, there was an inverse correlation between the stability of RNA polymerase-promoter complexes and in vivo activity for the Ball29 series.

Effect of negative supercoiling of template DNA on in vitro transcription of promoter constructs.

DNA bearing each of the promoter constructs was inserted into the plasmid vector pUC8 in the same orientation. Purified plasmid DNA of each clone was used as template for in vitro transcription. The plasmid templates were utilized either in their natural negative supercoiled state or in a form linearized by the restriction enzyme ScaI. E. coli pUC8 DNA is a very poor template for purified B. subtilis RNA polymerase which contains saturating levels of the delta subunit. Typically, the level of transcription from pUC8 DNA was less than 20% of that observed with the least efficient promoter construction used in this study, the Alu156 -34 deletion. The results presented in Fig. 5 were corrected for transcription from pUC8 for each RNA polymerase concentration.

The ability of the different Ball29 promoter constructs to direct transcription from a linear template as a function of RNA polymerase concentration is demonstrated in Fig. 5A. There was a direct correlation between RNA polymerase binding to a promoter construct in the competition binding assay and the level of RNA synthesis measured in the in vitro transcription assay. The best template contained the Ball29 hybrid promoter. The Ball29 and Ball29 extended promoters were also effective in vitro. The Ball29 -36 deletion promoter template gave about two-thirds the level of transcription as the Ball29 promoter. The poorest template in this series of promoters was the Ball29 -34 deletion promoter which demonstrated less than 15% of the Ball29 activity at the greatest RNA polymerase concentration tested.
When the same plasmid templates were used in a negatively supercoiled form (Fig. 5C), similar results were observed with two exceptions. The Ball29 hybrid promoter template was less effective relative to the Ball29 promoter in this form. The level of transcription from the Ball29 -34 deletion promoter was elevated four fold relative to the other promoters.

B. subtilis RNA polymerase effectively utilized the Alul56 and Alul56 hybrid promoters when present on a linear DNA fragment (Fig. 5B). None of the linear DNA fragments containing the Alul56 mutant promoters lacking the upstream DNA region served as productive templates. The Alul56 -36 deletion promoter allowed less than one-tenth the transcription as Alul56. These results are consistent with the measurements of RNA polymerase binding and in vivo promoter function.

When the Alul56 promoter series were tested for their ability to promote transcription from a negatively supercoiled template, there was a dramatic increase in the level of transcription from promoter constructions missing the upstream DNA region relative to linear templates (Fig. 5D). While Alul56 and Alul56 hybrid promoters were relatively unaffected by the DNA conformational change, there was conservatively a 6-10 fold increase in promoter activity for promoter lacking the upstream DNA region. In this in vitro assay, the negative supercoiling of the template was able to compensate, in part, for the absence of the upstream sequences.

Electrophoretic mobilities of promoter-containing DNA fragments.

DNA fragments containing either the Alul56 or Ball29 promoters were ligated with pBR322 digested with the restriction enzyme EcoRI. Purified pBR322 and plasmids possessing each of the promoter DNA fragments were linearized with one of a series of restriction enzymes. The linear DNA molecules were electrophoresed through polyacrylamide gels as described in the experimental procedures. The apparent molecular weight of each band was
estimated using lambda DNA digested with HindIII as molecular weight markers. The data in Table IIIA are presented as the ratio of apparent molecular weight determined by polyacrylamide gel electrophoresis to the actual molecular weight derived from the DNA sequence. The insertion of the promoter containing DNA fragments into pBR322 resulted in the altered electrophoretic mobility of the linear DNA. The Alu156 promoter containing plasmid digested with PstI displayed an apparent molecular weight 43% greater than the actual molecular weight. When the Bal129 promoter plasmid was linearized with the same enzyme, its apparent molecular weight was 40% greater than the actual molecular weight. Similar observations were made when the plasmids were digested with SalI. The apparent molecular weight of pBR322 was only 3% greater than the actual molecular weight under the same conditions. The small changes in apparent molecular weight of pBR322 were consistent with the work of Stellwagen (1983).

Estimates of molecular weight based on the electrophoretic mobilities of small, promoter-containing DNA fragments in polyacrylamide also deviated from the actual molecular size (Table IIIB). The Alu156 and Bal129 promoter DNA fragments routinely migrated at a rate consistent with a DNA molecule 20-22% larger than their actual size. Promoter constructs in which the polyadenine-containing upstream DNA sequences had been deleted migrated less abnormally. The discrepancy between apparent and actual molecular weight was further reduced by the replacement of upstream DNA sequences with non-promoter DNA in the Alu156 extended and Bal129 extended promoters. The abnormally slow polyacrylamide gel mobility of the Alu156 and Bal129 promoter DNA fragments may be indicative of altered DNA conformation. DNA sequences responsible for the altered mobility are contained, in part, in the DNA upstream from the -35 region.
DISCUSSION

Despite similarities in the nucleotide sequences of the Alul56 and Ball29 promoters (i.e. they share 67% homology between nucleotides -44 and +1, Fig. 2), the deletion of DNA immediately upstream from the -35 region affected each promoter differently. For this reason, the results obtained with each promoter will, initially, be discussed separately.

The Alul56 promoter. In general, efficient utilization of the Alul56 promoter was dependent on the DNA upstream from the -35 region. When the upstream DNA from the Ball29 promoter was substituted for the analogous DNA in the Alul56 promoter, the hybrid retained two-thirds of the original activity in vivo. However, the deletion of upstream DNA from the Alul56 promoter resulted in a 10 fold decrease in promoter function. In addition, relative to the original promoters, DNA fragments with the upstream deletion displayed reduced binding by RNA polymerase in competition binding assays. These results indicated that the formation of RNA polymerase-promoter complexes was influenced by the DNA upstream from the -35 region.

The results of in vitro transcription from linear DNA templates mimicked those obtained in vivo; there was a 10 fold decrease in transcription when the upstream DNA was deleted. The loss of promoter function for Alul56 promoters lacking the upstream DNA was partially restored if the DNA templates were negatively supercoiled. The mutants Alul56 -36 deletion and Alul56 extended, which differed in nucleotide sequence upstream from -41, displayed elevated transcription from supercoiled templates indicating that this effect was not a function of the sequence used to replace the polyadenine-containing upstream DNA. It is possible that the untwisting due to the negative supercoiling of these templates directly compensated for the loss of the upstream DNA. The untwisting of the DNA helix is a mandatory step in the formation of the open promoter complex. Since plasmids isolated using CsCl density gradients may be
more untwisted than those found in vivo (Sinden, et al., 1980), it is difficult to relate the in vitro transcription assays from supercoiled templates to in vivo CAT expression without further investigation.

The Ball29 promoter. The Ball29 promoter shares the elements of conserved DNA sequence with the consensus promoter sequences derived for gram-positive bacteria (Graves and Rabinowitz, 1986) and for the major E. coli RNA polymerase (Hawley and McClure, 1983; Rosenberg and Court, 1979; Siebenlist, et al., 1980; Harley and Reynolds, 1987). Despite this high homology with the consensus sequences, this promoter displayed 2 fold lower in vivo activity than the Alu156 promoter. The deletion of upstream DNA from the Ball29 promoter actually increased in vivo promoter function. The upstream DNA essential for the efficient function of the Alu156 promoter reduced Ball29 promoter activity in vivo.

Based on RNA polymerase binding assays, the Ball29 promoter has a much greater affinity for RNA polymerase than the Alu156 promoter. In dissociation experiments, the half-life of the Ball29 promoter was at least 40 fold longer than the Alu156 promoter. It is possible that the binding between the RNA polymerase and the Ball29 or Ball29 hybrid promoter is so tight that the clearance of the RNA polymerase from the promoter after initiation is impaired. By this reasoning, a mutation that decreased the affinity of the RNA polymerase for this promoter could be expected to have little effect or actually enhance in vivo promoter function (e.g. if the increase in promoter clearance was more significant than the decrease in promoter binding). Results similar to this were obtained. The Ball29 -36 deletion promoter displayed half again the level of activity as the Ball29 promoter.

The competition binding assay confirmed that, similar to Alu156, the loss of the upstream DNA from the Ball29 promoter decreased the affinity of RNA polymerase for this site. Dissociation studies demonstrated half-lives for
Ball29 promoters lacking upstream DNA on the order of one-tenth those determined for the Ball29 and Ball29 hybrid promoters. In fact, the Ball29 hybrid promoter, which allowed only low level expression in vivo, consistently displayed the longest half-life in dissociation studies. This supports the idea that the low in vivo activities of the Ball29 and Ball29 hybrid promoters are due to a reduced ability of the RNA polymerase to leave the promoter following initiation and that the upstream DNA contributes to this tight binding.

Altered conformation of promoter DNA. The electrophoretic mobilities of linear DNA fragments containing the Ball29 or Alul56 promoter were examined. The 186 base pair Alul56 and 162 base pair Ball29 containing DNA fragments migrated at a size about 20% larger than predicted. When small DNA fragments containing these promoters were inserted in pBR322, the apparent molecular weight of the entire molecule increased 40% over the actual size. The ability of these small DNA fragments to impart aberrant electrophoretic mobilities on larger DNA molecules and the presence of runs of adenines with a 10.5 base pair periodicity in the DNA immediately upstream from the -35 region are consistent with the existence of a region of curved DNA (Hagerman, 1985; Koo and Crothers, 1987; Tifonov, 1985). Based on sequence predictions involving the length of the runs of adenines and the spacing that separates them, the upstream region from Alul56 would possess greater sequence dependent curvature than that from Ball29.

Several models have been proposed to explain the stimulation of transcription by DNA upstream of the -35 region (Bossi and Smith, 1984; Lamond and Travers, 1983; Plaskon and Wartell, 1987). In one model, it is proposed that the curved DNA upstream of the -35 region acts as an RNA polymerase binding site to maintain a locally high concentration of enzyme, thus, facilitating binding to the promoter. The findings presented in this report
are inconsistent with this model. Specifically, it was shown that the replacement of the curved upstream DNA of the Ball29 promoter with other DNA resulted in a 10 fold decrease in the stability of preformed RNA polymerase-promoter complexes. This suggests that the curved DNA influences the interaction between the RNA polymerase and DNA after the initial binding (i.e. after the formation of the initial closed promoter complex). In addition, under the conditions used in the nitrocellulose filter binding assay, no filter retainable complexes were detected when the holoenzyme (core-\(\sigma^4\)) was incubated with fragments containing the DNA upstream from -33 for either the Ball29 or Alu156 promoter (data not shown).

The results described in this report are consistent with a model that involves the enhanced binding of RNA polymerase to the promoter due to additional RNA polymerase-DNA interactions. One version of this model (Plaskon and Wartell, 1987) predicts the interaction of the RNA polymerase with DNA upstream of the -35 region which is made accessible to enzyme binding through curvature of the DNA. These additional RNA polymerase-DNA interactions could contribute to the unstacking of the paired bases prior to the formation of the open promoter complex (Rozenberg, et al., 1982; Roe, et al., 1984; Buc and McClure, 1985). The curved upstream DNA would enhance transcription from promoters for which open complex formation is rate limiting. This would appear to be the case for the Alu156 promoter, the wild-type promoter with the weakest affinity for RNA polymerase. In contrast, transcription from the Ball29 promoter appeared to be limited at the step of promoter clearance and not at the level of open promoter complex formation. This could explain why deletion of the upstream DNA and loss of potential RNA polymerase-promoter interactions did not result in the loss of promoter activity. The mutant promoters described in this work will allow several aspects of the above models to be
experimentally tested with assays designed to probe the specific RNA polymerase-promoter complexes formed during transcription initiation.
TABLE I
Promoter-directed chloramphenicol acetyltransferase activity.

<table>
<thead>
<tr>
<th>Promoter fragment</th>
<th>CAT specific activity$^a$</th>
<th>Percent of original promoter activity$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alu156</td>
<td>2.6</td>
<td>100</td>
</tr>
<tr>
<td>Alu156 Hybrid</td>
<td>1.7</td>
<td>65</td>
</tr>
<tr>
<td>Alu156 -36 Deletion</td>
<td>0.2</td>
<td>8</td>
</tr>
<tr>
<td>Alu156 -34 Deletion</td>
<td>0.1</td>
<td>4</td>
</tr>
<tr>
<td>Alu156 Extended</td>
<td>0.1</td>
<td>4</td>
</tr>
<tr>
<td>Ball29</td>
<td>1.3</td>
<td>100</td>
</tr>
<tr>
<td>Ball29 Hybrid</td>
<td>0.5</td>
<td>38</td>
</tr>
<tr>
<td>Ball29 -36 Deletion</td>
<td>2.0</td>
<td>154</td>
</tr>
<tr>
<td>Ball29 -34 Deletion</td>
<td>1.2</td>
<td>92</td>
</tr>
<tr>
<td>Ball29 Extended</td>
<td>1.4</td>
<td>108</td>
</tr>
</tbody>
</table>

$^a$ Chloramphenicol acetyltransferase specific activity expressed as micromoles chloramphenicol acetylated per minute per milligram protein at 25°C. Average CAT specific activity was determined from three independent transformants containing the correct pPL703-promoter transcriptional fusions. The values were corrected for background measurements with pPL703 containing B. subtilis 1A510.

$^b$ The original promoters are designated Alu156 and Ball29.
### TABLE II

Dissociation of RNA polymerase from promoter-containing DNA fragments.

<table>
<thead>
<tr>
<th>Promoter fragment</th>
<th>Half-life$^a$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alu156</td>
<td>1.2</td>
</tr>
<tr>
<td>Alu156 hybrid</td>
<td>1.2</td>
</tr>
<tr>
<td>Alu156 -36 deletion</td>
<td>1.4</td>
</tr>
<tr>
<td>Alu156 -34 deletion</td>
<td>0.4</td>
</tr>
<tr>
<td>Alu156 extended</td>
<td>1.4</td>
</tr>
<tr>
<td>Ball29</td>
<td>52.8</td>
</tr>
<tr>
<td>Ball29 hybrid</td>
<td>59.4</td>
</tr>
<tr>
<td>Ball29 -36 deletion</td>
<td>3.1</td>
</tr>
<tr>
<td>Ball29 -34 deletion</td>
<td>2.5</td>
</tr>
<tr>
<td>Ball29 extended</td>
<td>5.2</td>
</tr>
</tbody>
</table>

$^a$RNA polymerase-promoter DNA complexes were formed at an enzyme/DNA weight ratio of 10 for 10 min at 37°C. Dissociation of RNA polymerase from the complexes was measured in the presence of single stranded M13 DNA as described under "Experimental Procedure." The half-life values presented are the average of 3-6 determinations in which the five promoter fragments of each series were analyzed in the same reaction.
Table III

Apparent molecular weight of promoter-containing DNA fragments.

<table>
<thead>
<tr>
<th>DNA length</th>
<th>Apparent molecular weight(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Actual molecular weight</td>
</tr>
<tr>
<td></td>
<td>base pairs</td>
</tr>
<tr>
<td>A. Linear plasmid DNA</td>
<td></td>
</tr>
<tr>
<td>pBR322-PstI</td>
<td>4363</td>
</tr>
<tr>
<td>pBR322-SalI</td>
<td>4363</td>
</tr>
<tr>
<td>pBR322/Alu156-PstI</td>
<td>4549</td>
</tr>
<tr>
<td>pBR322/Alu156-SalI</td>
<td>4549</td>
</tr>
<tr>
<td>pBR322/Bal129-PstI</td>
<td>4525</td>
</tr>
<tr>
<td>pBR322/Bal129-SalI</td>
<td>4525</td>
</tr>
<tr>
<td>B. DNA fragments</td>
<td></td>
</tr>
<tr>
<td>Alu156</td>
<td>186</td>
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<tr>
<td>Alu156 -36 deletion</td>
<td>85</td>
</tr>
<tr>
<td>Alu156 -34 deletion</td>
<td>75</td>
</tr>
<tr>
<td>Alu156 extended</td>
<td>173</td>
</tr>
<tr>
<td>Bal129</td>
<td>162</td>
</tr>
<tr>
<td>Bal129 -36 deletion</td>
<td>119</td>
</tr>
<tr>
<td>Bal129 -34 deletion</td>
<td>109</td>
</tr>
<tr>
<td>Bal129 extended</td>
<td>207</td>
</tr>
</tbody>
</table>

\(^a\)Linear DNA fragments containing the indicated promoter sequences were electrophoresed on 4% (A) or 8% (B) polyacrylamide gels using a TBE buffer system as described under "Experimental Procedure." Determination of apparent molecular weights was based on electrophoretic mobilities using lambda DNA-HindIII (Part A) or pBR322-MspI and pBR322-HaeIII (Part B) molecular weight markers.
Fig. 1. Nucleotide sequence immediately upstream of the -35 region for each promoter in the Alul56 and Ball29 series. The boxed area encompasses the conserved nucleotide sequence of the -35 region for each promoter. The promoter-derived DNA for the deletion constructions terminates at the -35 region; the terminal EcoRI restriction sites originate from the vector. The length, in base pairs, of each fragment is listed in parentheses. These DNA fragments were employed in the RNA polymerase binding assays described under "Experimental Procedure".
Fig. 2. Nucleotide sequence of the Alu156 and Ball29 promoter-containing DNA fragments. Bases representing the +1 transcription start site, the −35 region, and the −10 region of the Alu156 and Ball29 promoters are in bold type. The runs of adenines in the DNA upstream from the −35 region are underlined.

Ball2 9 G A A T T C C C C G G A T C C G T C A

-70 -60 -50 -40 -30 -20 -10 +1


+10 +20 +30 +40 +50 +60


Ball29 G A A T T C C C G G A T C C G T C A

-70 -60 -50 -40 -30 -20 -10 +1


+10 +20 +30 +40 +50 +60


Fig. 3. Relative affinity of B. subtilis RNA polymerase for Ball29 and AluL56 promoters. The competition binding assay measured the fraction of promoter-containing DNA retained on a nitrocellulose membrane filter as a function of RNA polymerase concentration. At each RNA polymerase concentration, the reaction contained equal amounts of DNA fragments containing the Ball29 promoter (●), the Ball29 hybrid promoter (▲), the AluL56 promoter (○), and the AluL56 hybrid promoter (△). RNA polymerase binding was quantitated as described under "Experimental Procedure".
Fig. 4. Relative affinity of B. subtilis RNA polymerase for DNA fragments containing promoters with altered upstream DNA. The competition binding assay was used with DNA fragments carrying the promoters from the Ball29 series (A) or the Alu156 series (B). At each RNA polymerase concentration tested, the reactions contained equal amounts of DNA fragments with the original promoter (●) and four promoters with altered upstream DNA. Within each series, promoter containing DNA fragments included the hybrid promoter (▲), the extended promoter (△), the -36 deletion promoter (□), and the -34 deletion promoter (○). Quantitation of individual DNA fragments was described under "Experimental Procedure".
Fraction of DNA Bound
Fig. 5. **In vitro transcription from supercoiled and linear DNA templates.** Incorporation of nucleoside triphosphates was measured as a function of RNA polymerase concentration. Transcription from linear DNA templates for each promoter construct in the Ball29 series (A) and Alu156 series (B) and from supercoiled DNA templates for the Ball29 (C) and Alu156 (D) series of promoters were measured as described under "Experimental Procedure". The different symbols refer to the same promoter constructions as outlined in the legend to Fig. 4.
CHAPTER II

Effects of Incremental Displacement of Upstream DNA on Promoter Function in *Bacillus subtilis*. 
INTRODUCTION

Altered conformation of DNA has been implicated in regions of DNA that are required for the regulation of replication and transcription. These regions possess sequence-dependent curvature or are bent or looped by regulatory proteins.

Sequence-dependent curvature for the kinetoplast minicircle DNA from trypanosomes (Marini et al., 1982) was detected by abnormally slow migration rates of restriction fragments on polyacrylamide gels. Although the exact mechanism of DNA curving is still unknown, it is clear that curved regions examined to date are characterized most often by runs of two to six adenines which are spaced every 10-11 bp, or one helical turn apart. The extent of curvature imparted by regularly phased adenines has been determined (Marini et al., 1982; Wu and Crothers, 1984; Koo et al., 1986; Hagerman, 1984, 1985, 1986) for a number of natural and synthetic molecules by comparing the actual molecular weight to the apparent molecular weight calculated from their electrophoretic migration in polyacrylamide gels. Estimates of curvature have been made based on the ability of the ends of synthetic curved molecules to circularize and be ligated into closed circles (Ulanovsky et al., 1986; Zahn and Blattner, 1987). The curvature for a trypanosome kinetoplast 200 bp linear DNA fragment has also been demonstrated by electron microscopy (Griffith et al., 1986, Launden and Griffith, 1987).

DNA curving has been suggested to be important in the wrapping of chromatin by histones to form nucleosomes (Mengeritsky and Trifanov, 1984; Zhurkin, 1983). Sequences similar to those found in curved kinetoplast DNA have been found in the origin of replication of the
phages lambda (Zahn and Blattner, 1985) and ΦX174 (Sargosti et al., 1982), Simian virus 40 (Ryder et al., 1986), as well as plasmids pBR322 (Stellwagon, 1983), pR6K (Mukherjee et al., 1985) pT181 (Koepsel and Kahn, 1986), F plasmid (Tokino et al., 1986) and a yeast autonomously replicating sequence (Snyder et al., 1986) rrrBP. For several of these examples, the curved DNA appears to aid the binding of a replication protein which then functions in altering the DNA conformation by bending the DNA. Curved DNA may also have a role in the structuring of DNA for site-specific recombination as in the lambda attP site (Leong et al., 1985) and the att sites of phi-80 and P22 phages (Ross et al., 1982; Ross and Landy, 1982).

Curved DNA has been detected upstream of the ompF (Mizuno, 1987; Lamond and Travers, 1983), tyrT, promoters of E. coli, and the hisR promoter in Salmonella (Bossi and Smith, 1984). Deletion of these regions or changes which reduced curvature also resulted in decreased transcription from these promoters. A run of adenines (i.e. a polyadenine group) at -45 has now been identified as a conserved feature among a subset of E. coli promoters (Galas et al., 1985). Those containing this region of upstream curvature are generally associated with highly expressed genes (Plaskon and Wartell, 1987). This -45 region appears to be more highly conserved among promoters from gram-positive bacteria, such as B. subtilis (Graves and Rabinowitz, 1986). Evidence has been presented to suggest that the absence of this region may constitute one reason why many strong E. coli promoters are not efficiently utilized by the major B. subtilis RNA polymerase (Peschke et al., 1985). A B. subtilis promoter which is recognized by minor forms of the RNA polymerase holoenzyme (i.e.,
those containing $\sigma^{37}$ and $\sigma^{32}$ is dependent on upstream regions which are rich in adenines and thymines and resemble those DNAs which have been shown to contain curvature (Banner et al., 1983). Recently, we reported the presence of three successive runs of adenines at -45, -55 and -65 in an early gene promoter from the B. subtilis phage SP82 (McAllister and Achberger, 1988). The promoter, designated Alu 156, showed marked dependence on these upstream sequences for efficient promoter function. Loss of these regions resulted in at least a 10 fold decrease in in vivo CAT activity, in vitro transcription, and binding of RNA polymerase in vitro. These upstream regions were shown to display DNA curvature as determined by abnormally slow electrophoretic migration rates in polyacrylamide gels. The polyadenine regions direct the curve toward the same side of helix as the -35 region, the side of the helix for which the majority of RNA polymerase:promoter interactions have been detected using chemical probes.

This report describes the relationship between the precise structural orientation of these polyadenine regions relative to the promoter proper and their function as a cis-acting stimulatory element for transcription from the Alu156 promoter. The structure/function relationship was investigated using mutants created by the insertion of oligonucleotides between the polyadenine regions and the -35 region.
EXPERIMENTAL PROCEDURE

Materials. Restriction enzymes and DNA modifying enzymes were purchased from Bethesda Research Laboratories or New England Biolabs, Inc. Dideoxy- and deoxynucleotides were obtained from Pharmacia, Inc. Dideoxyadenosine 5'- (a-thio) triphosphate and [5,6-^H] Uridine 5'- triphosphate were purchased from New England Nuclear. Nucleoside triphosphates were obtained from Sigma Chemical Co. All other reagents were of the highest purity available.

Synthesis and purification of oligonucleotides. The complimentary oligonucleotides 5' CCGGATGGATT 3' and 5' AATCGATCOGG 3' were synthesized using an Applied Biosystems automated DNA synthesizer model 380A. Purification (Itakura et al., 1984) was performed by a modified procedure using a Speed-Vac concentrator (Savant) for concentration and evaporation steps. The oligonucleotides were first released from the solid support column by an overnight treatment at 55°C with concentrated ammonium hydroxide. The tritylated DNA was then separated from the nontritylated (incomplete) molecules by reverse phase chromatography using a Sep-pak C18 column (Waters Associates) and a 10% and 30% step gradient of acetonitrile in triethylammonium bicarbonate (TEAB) buffer pH 7.4. The trityl groups were then removed with 80% glacial acetic acid and the oligomers were gel purified on a 20% polyacrylamide gel containing 8M urea. The DNA bands were identified by U.V. shadowing the gel over a TLC silica gel plate containing a fluorescent dye. The appropriate gel regions were excised with a razor blade, minced, and eluted overnight in TEAB at 37°C on a rotary shaker. Acrylamide was removed by centrifugation and filtration through a glass fiber filter (Schleicher and Schuell), and
the sample was again subjected to reverse phase chromatography. The sample was then evaporated, rehydrated in water, quantitated by U.V. absorption and equal amounts of each strand were hybridized (Maniatis et al., 1982) using 4x SSC buffer for one each hour at 62°C, 45°C, and 37°C. The same techniques were used to purify a six base oligomer (5' CGTCCA 3') upon which self-hybridization generated a symmetrical double stranded molecule with 5' unpaired termini of two bases. This molecule contains the same sequence regardless of the orientation in which it is inserted.

**Construction of mutant promoters.** A 156 bp AluI fragment containing the Alu156 promoter had previously been blunt-end ligated into the HincII sites of M13 mp7. This destroyed the two vector HincII sites so that the only remaining HincII site was at the -33 position of the Alu156 promoter. This site was used to insert the double-stranded, blunt-ended 11-mer which had been 5' phosphorylated using T4 polynucleotide kinase and ATP. Insertion of a single oligonucleotide in the correct orientation restored the original thymines present at -34 and -35 in the promoter. We then inserted BamHI fragments of Alu156 and the +11 mutant from M13 mp7 into BamHI digested pUC8 in which the linker SalI site had been destroyed. The Alu156-pUC8 constructs were transformed into E. coli JM83 and the +11-pUC8 constructs were transformed into the E. coli dam- strain GM2163. We then determined which Alu156-pUC8 constructions contained the promoter in the correct orientation (i.e., -35 region oriented toward the EcoRI linker site) by sizing the restriction fragments from a
digestion with EcoRI, HincII, and PstI on polyacrylamide gel. The orientation of the +11 constructs was confirmed by the sizing of EcoRI, ClaI restriction fragments on a polyacrylamide gel.

The +6 mutant was then constructed by the deletion of the five bp region between the MspI and the ClaI sites on the eleven base insert of the +11-pUC8 construction. This was accomplished by isolation of the upstream portion of the promoter as a gel purified BamHI, MspI restriction fragment, and the downstream portion of the promoter as a ClaI, PstI restriction fragment and ligating the compatible MspI and ClaI termini of these fragments in a three component ligation with pUC8 DNA which had been digested with BamHI and PstI. The +13 mutant was formed by making in the ClaI termini of the +11 construction flush with DNA polymerase I large fragment and dCTP and dGTP and religation of the resulting blunt ends. The +15 mutant was obtained by digestion of the +11 promoter with BspMII and making the four base 5' termini flush DNA polymerase I large fragment and dCTP and dGTP. The +17 mutant was created by the insertion of a single copy of the 6-mer described earlier into the +11-pUC8 promoter construction which had been digested with ClaI. This generated an AccI and a Sall site, which were each digested and made flush as described above to give the +19 and +21 insertion mutants, respectively. The +21 mutant contained a BspMII site which was filled in to produce the +25 mutant. The +9 mutant was constructed by polyacrylamide gel purification of the upstream portion of the +11 promoter as a BamHI, MspI fragment and the downstream portion of the +17 promoter as a TaqI, PstI fragment and joining the TaqI and MspI compatible termini in a three component ligation with pUC8 DNA which had been digested with BamHI and PstI.
Lastly the +29 mutant was created in an analogous way using pUC8 that had been digested with EcoRI and PstI and the upstream EcoRI, TaqI generated fragment from the +17 mutant promoter and the MspI, PstI generated downstream fragment of the +21 promoter. It should again be noted that all cloning where ClaI or TaqI digestions were needed were carried out using the dam- E. coli strain GM2163. Mutant promoters were confirmed by a combination of restriction digest analysis and nucleotide sequencing (Sanger et al., 1977).

In vivo CAT expression. Transcriptional fusions were created between each promoter construction described above and the chloramphenicol acetyltransferase, CAT, gene of pPL703 (Duvall et al., 1983) as reported previously (McAllister and Achberger, 1988). These recombinant plasmids were transformed into B. subtilis IA510 (Bacillus Genetic Stock Center, The Ohio State University; Ostroff and Pene, 1984) protoplasts by a modification (McAllister and Achberger, 1988) of the method of Chang and Cohen (1979). Selection and screening of transformants, CAT assays (Shaw, 1975) and protein concentration determinations (Bradford, 1976) were performed as previously described (McAllister and Achberger, 1988).

RNA polymerase binding assays. B. subtilis Eo436 RNA polymerase was isolated (Achberger and Whiteley, 1981; Spiegelman et al., 1978) as described previously (McAllister and Achberger, 1988). The RNA polymerase dependent retention of promoter-containing DNA fragments on nitrocellulose membrane filters was based on the method of Jones et al. (1977) with modifications (McAllister and Achberger, 1988). The
polymerase for various of promoter-containing DNA fragments as a function of RNA polymerase concentration.

The competition binding assay requires that all promoter-containing DNA fragments be separable by electrophoresis. Since the wild type Alu 156 promoter and ten mutant promoters differ in size by very small increments (e.g., 2 bp in some cases), it was not possible to separate a mixture containing all of them on a 6% polyacrylamide gel. Each promoter could be excised from the pUC8 vector with BamHI, with EcoRI and PstI, or with EcoRI and HindIII. The latter two digests give promoter fragments which are 21 and 29 bp longer, respectively, than the former. Using various digests for each promoter we were able to construct a number of groups of promoters, each containing the Alu 156 wild type promoter and three or four mutant promoters, that were resolvable on 6% polyacrylamide gels. In each 0.4 ml RNA polymerase binding assay 1.5 µg of digested plasmid was added for each promoter tested. The pUC8 vector contains no promoters which are efficiently bound or transcribed by B. subtilis Eσ43σ RNA polymerase, and was included in the binding reaction to minimize non-specific binding, such as end binding (Melancon et al., 1983), to the test fragments.

Polyacrylamide gel electrophoresis. Electrophoresis to separate small promoter containing DNA fragments for densitometric quantitation of filter binding studies employed 6% (60:1 monomer to bis ratio) polyacrylamide gels. Electrophoresis conditions were 7 volts/cm for 5 hr at room temperature. For fragment mobility determinations, 10% (60:1 monomer to bis ratio) polyacrylamide gels were used. Electrophoresis conditions for fragment mobility determinations were
Electrophoresis conditions for fragment mobility determinations were made at 6.5 volts/cm (also limited to 24 milliamperes) for 6 hrs, 11 hrs and 21 hrs, respectively at 65°C, 23°C, and 5°C. All gels utilized a TBE buffer system (89 mM Tris, 89 mM boric acid, 2.5 mM disodium EDTA, pH 8.3). For fragment mobility determinations a peristaltic pump was used to recycle running buffer between the upper and lower reservoirs at a rate of 10 ml/min. For routine screening of constructions and for preparative isolation of promoter containing DNA fragments, either 6% or 10% gels were employed at 10 volts/cm (and limited to 25 milliamperes).

**General techniques.** Transfection of *E. coli* JM101 and transformation of *E. coli* JM83 was accomplished with CaCl2 treated cells (Maniatis et al., 1982). Plasmid DNA and M13 replication form was isolated using the alkaline lysis method (Birnboim and Doly, 1979) and CsCl gradient density gradient centrifugation in the presence of ethidium bromide. Manufacturers recommendations were followed in the use of restriction enzymes and DNA modifying enzymes. Following electrophoresis and staining with ethidium bromide, DNA fragments to be purified were excised from polyacrylamide gels with a razor blade, minced, and eluted overnight at 37°C in TBE buffer. The DNA was concentrated and further purified using DE52 (Whatman) chromatography and ethanol precipitation. CsCl purified vector DNA which was digested with more than one restriction enzyme was electrophoresed on a 0.6% agarose gel to separate it from the excised linker, visualized by U.V. shadowing over a fluorescent screen, excised with a razor
blade, and electroeluted from the gel slice into a dialysis bag. The DNA-containing eluent was then phenol extracted, ether extracted, and ethanol precipitated.
RESULTS

The Alul56 promoter possesses polyadenine-induced DNA curvature upstream of the -35 region (Figure 1). Previous studies support the idea that the curved DNA is required for efficient promoter utilization in B. subtilis (McAllister and Achberger, 1988). To ascertain the restrictions for the stimulation of transcription by curved DNA, a series of mutant promoters was constructed by the insertion of synthetic, double-stranded oligonucleotides of various lengths between the -35 region and the downstream most polyadenine region of Alul56. These incremental insertions served a dual purpose. They displaced the polyadenine regions from the -35 region and the rest of the promoter by discrete distances along the DNA helix. They also served to rotate the polyadenine-induced curved DNA around the helix with respect to the -35 region. Figure 2 shows the relevant sequence of these mutant promoters as well as the distance and rotational separation of the -35 region and the polyadenine regions for each mutant. Assuming that the insert DNA is in B form, each 10.5 bp insertion would result in a linear displacement of 3.4 nm and a rotational displacement of 360 degrees (i.e., one full helical turn).

Electrophoretic mobilities of promoter containing DNA fragments. It was previously determined that the Alul56 promoter fragment migrated in 8% polyacrylamide gels at a rate consistent with a molecule 22% larger than its actual size. Deletion or replacement of the upstream region significantly reduced but did not entirely eliminate this aberrant mobility. This suggested that the majority of the curvature of this promoter could be attributed to the upstream polyadenine regions, but that the promoter sequence downstream from
the HincII site at -33 also exhibited some curvature. If there is more than one curve, the rotation of the upstream DNA relative to the -35 region should significantly affect the structure of the promoter fragment. To test for altered DNA conformations, the promoters constructed for this study were excised from pUC8 with EcoRI and PstI restriction endonuclease and electrophoresed on 10% acrylamide gels at three different temperatures. Figure 3 is a graphical depiction of the apparent to actual molecular weight ratios as a function of the length of the insert in each promoter mutant. It has been noted that the abnormal electrophoretic mobility of curved DNA returns to normal at elevated temperatures (Marini et al., 1984; Bossi and Smith, 1984). Consistent with this observation, at 65°C, DNA fragments containing the various promoters exhibited electrophoretic migration rates close to that predicted from the fragment lengths. The findings that there was little difference in mobilities at 23°C and 5°C and that even at 65°C aberrant electrophoretic mobility was still observed were consistent with the existence of a highly stable, curved DNA molecule.

A striking observation is that the apparent to actual molecular weight ratio does not remain constant, as would be predicted for a straight linear molecules of increasing molecular weight. The flat line progression would also be expected if the molecule exhibited curvature on only one side of the point of oligonucleotide insertion at -36. The cyclical pattern would only be observed if there is a region of curved DNA on both sides of the rotation point. The observed cyclical pattern has a periodicity of approximately one helical turn as seen by the apparent to actual molecular weight ratio values which reach a maximum at the +6 and +17 fragments and a minimum
at the +15 and +25 promoters for gels run at 23°C. The +6 and +17 fragments should have the polyadenine regions on the opposite side of the helix relative to the wild type and +11 and +21 mutants. It has been noted that decreasing end-to-end distance directly correlates with decreasing electrophoretic migration rates for DNA fragments in polyacrylamide gel (Wu and Crothers, 1984). This means that when two regions of curved DNA are present on a single molecule, the electrophoretic mobility would be retarded most when the two curves are in the same orientation (i.e., the DNA molecule is U-shaped). The electrophoretic mobility would be closest to normal when the two curves are directionally opposed forming an S-shaped molecule. Using this reasoning the regions of curved DNA in the wild-type Alu 156 and +11 and +21 mutant promoters would be directionally opposed.

If the three polyadenine regions were the only regions of DNA curvature upstream from the -35 region, one would expect to see the points for each curve in Figure 3 arranged so that a single sine curve could be drawn through them. The data however show a prominent skew on the right side of each peak. The 5°C migration pattern reveals an even more pronounced shoulder in this region. The most likely explanation for these patterns is the presence of a third region of curvature whose stability is enhanced at 5°C. The presence of such a region would explain the observed skewing as additive effect of two sine curves with the second having a smaller amplitude, presumably due to a lesser degree of curvature. The cycling of the two sine curves indicate the two DNA curves are offset by approximately two to four bp (68 to 137 rotational degrees).
In vivo expression from Alul56 promoter constructions. Transcriptional fusions were established between each of the promoters and the chloramphenicol acetyltransferase (CAT) gene carried on the promoter cloning vector pPL703. The levels of CAT specific activity measured for each promoter construction in B. subtilis are presented in Figure 4A. Previously, it was demonstrated that Alul56 derivatives in which the upstream polyadenine-containing DNA had been deleted gave CAT specific activities which were less than 10% of the wild-type Alul56 (McAllister and Achberger, 1988). From the data in Figure 4A, it can appeared that the effect of the dependent on the rotational orientation of this region with the promoter proper. The mutant constructions which allowed the highest levels of CAT expression were the promoters in which the polyadenine regions were rotated by 1 or 2 turns of the helix (e.g., +11 and +21) and thus, in the same rotational orientation as the wild-type Alul56 promoter. CAT specific activities decrease to a minimum at or very near the point at which the polyadenine regions would be on the opposite side of the DNA helix from the -35 region (+15, +25). The high CAT specific activity observed from the +6 and +9 mutant promoters may reflect a rotation independent effect based on the proximity of the polyadenine region to the promoter proper.

Effect of promoter mutations on in vitro binding of RNA polymerase. To ascertain if the changes in in vivo promoter function reflected differences in the affinity for RNA polymerase, the binding of purified B. subtilis (Eo438) RNA polymerase to the wild type and mutant promoters was examined. The wild-type promoter was allowed to compete with various combinations of three to four mutant promoters
for subsaturating levels of RNA polymerase. Equal molar amounts of each promoter were used in these filter binding assays. The results of these competition binding experiments are shown in Figure 4B.

The binding data agree remarkably well with the results from the in vivo expression assays. Maximum binding of RNA polymerase was observed for the wild type and the +11 and +21 mutants in which the rotational orientation of the upstream polyadenine regions relative to the -35 region is identical. The lowest levels of polymerase binding were observed for promoters in which these regions were aligned opposite the -35 region on the helix (i.e., +6, +17, and +25 or +29 mutants). This suggests that the rotational orientation of upstream and downstream curvature and not the linear displacement is important in producing a DNA conformation for maximal binding of RNA polymerase.

In the same set of experiments, when a mutant of Alu156 that lacked the upstream DNA, the Alu156 extended promoter (McAllister and Achberger, 1988) was used, it was bound by RNA polymerase one-half as efficiently as the weakest insertion mutant, the +15 mutant.

There are two apparent differences between the in vivo data and the in vitro binding data. The +6 mutant appears to bind RNA polymerase very poorly. If the polyadenine region can affect promoter function by its proximity to the promoter, this effect does not appear to be at the level of RNA polymerase binding. Second the +13 mutant exhibits very low CAT expression in vivo but appears to bind RNA polymerase very well. Separate experiments have demonstrated that this promoter is not deficient in initiation when compared to the other mutant promoters. It should be noted that no measurements of the rate of open promoter complex formation or the rate of initiation
have been made. It appears that the orientation of the curved DNA in the +13 mutant allows RNA polymerase binding but not effective transcription overall.

The RNA polymerase binding data presented in Figure 4B represent results from a single enzyme concentration in the competition binding assay. The data in Figure 4B has been normalized to the level of RNA polymerase binding observed for the Alu156 promoter-containing fragment which was present in each assay. An example of the competition binding assay is presented in Figure 5. In each case, DNA fragments containing the mutant promoters compete with the wild-type promoter for subsaturating amounts of RNA polymerase. Low recovery of weakly bound promoters was observed until the promoters with a high affinity for RNA polymerase were bound at their maximum level. Data for the Alu156-normalized binding presented in Figure 4B was generated at an enzyme/DNA weight ratio of 2.5 (e.g., the 15 μg point in Figure 5). It should be noted that the shape of the curve for Alu156 (Figure 5) in no way reflects the cooperative binding of more than one RNA polymerase molecule.

A technique that complements the comparison of in vivo gene expression and in vitro RNA polymerase binding is that of in vitro transcription.

**Effects of insertion mutagenesis of Alu156 on in vitro transcription.** The ability of the wild type and mutant promoters to direct transcription from linear and supercoiled pUC8-derivative templates was tested using the same RNA polymerase:DNA ratios as in the binding experiments. Supercoiled templates were used as isolated or were linearized with ScaI which cleaves the DNA approximately 900
bp downstream from the transcriptional start site of Alul56. B. subtilis E. coli containing saturating levels of the delta subunit utilizes E. coli pUC8 promoters very poorly. Background transcription rates for pUC8 with no promoter insert were less than 10% of those observed for the least efficient promoter used in the study, the +17 promoter, and the results shown in Figure 4C have been corrected for this background.

The in vitro transcription agree with the in vitro binding and in vivo CAT expression findings. Promoters which gave the most efficient UMP incorporation (Alul56, +11, and +21), also bound RNA polymerase better, and gave higher in vivo CAT expression. Again the promoters which gave the lowest transcription rates in vitro were those in which the polyadenine regions had been rotated opposite the -35 region on the DNA helix.

The differences seen in Figure 4C for the levels of transcription from linear vs supercoiled templates is primarily due to the different lengths of the respective transcripts. The mRNA synthesized from the linear transcripts is only 900 bases long, whereas transcription from the supercoiled template would be expected to give a transcript of 1600 bases in length if terminated efficiently at the bla gene terminator. The increased incorporation of UMP seen with the supercoiled templates seems consistent through the series of insertion mutants when compared to the levels from linear templates for each construction. The notable exceptions are the +15 and +25 insertion mutants. The partial untwisting of the negatively supercoiled DNA
template appears to stimulate transcription from mutant promoters in which the DNA conformation is not optimal for transcription initiation.
DISCUSSION

The evidence presented here corroborates and extends the earlier findings that the polyadenine regions upstream from the -35 region are required for efficient utilization of Alu156 by B. subtilis RNA polymerase (McAllister and Achberger, 1988). The present work demonstrated that orientation of the polyadenine regions on the same side of the helix is required for maximum stimulation of transcription by these upstream regions. It is interesting to note that the lowest in vivo CAT activity observed that of the +15 mutant still represents at least 12% of the wild type level. The average CAT expression for two different Alu156 derivatives in which the upstream polyadenine regions had been deleted represented only 6% of the wild type (McAllister and Achberger, 1988). In no case did the levels of RNA polymerase binding, in vitro transcription, or in vivo utilization for the insertion mutants of Alu156 decrease to the low levels observed when the upstream polyadenine regions were deleted. This suggests that while the rotational orientation of the curved DNA relative to the promoter proper is necessary to specify how efficiently the promoter is utilized by B. subtilis RNA polymerase, there seems to be some measure of transcriptional stimulation effected by these upstream regions that is independent of their rotational orientation.

The evidence that supports an effect of the linear displacement of curved DNA includes the high in vivo activity and low in vitro RNA polymerase binding of the +6 mutant. These data are consistent with a positive effect of having the polyadenine region within 11 bp of the promoter proper. Insertion mutants of less than 6 bp (e.g., +2 and +4 insertions) will be required to fully test this observation. In
addition, the stimulation of in vitro transcription of two promoters by negative supercoiling was somewhat dependent on the linear displacement of the upstream curved DNA. Negative supercoiling enhanced transcription from the +15 mutant promoter slightly but had a significant effect on the +25 mutant. It was previously demonstrated that transcription from Alu156 mutant promoters from which the curved, upstream DNA was deleted were strongly stimulated by negative supercoiling of the template while transcription from the wild-type Alu156 promoter was relatively unaffected (McAllister and Achberger, 1988). It appears that the proximity of the promoter to the curved DNA in the wrong orientation (i.e., the +6 and +15 mutants) cancels the effect of negative supercoiling. Only when the curved DNA is sufficiently displaced, as in the +15 mutant, or removed, as with the Alu156 -36 deletion mutant, would the effect of helix unwinding in negatively supercoiled DNA not be influenced by the altered conformation of DNA curvature.

**Location of sequence-dependent DNA curves.** The skewed nature of the right side of the peaks in Figure 3 and the enhancement of a shoulder in this area at 5°C indicated a third region of curvature which appears to be offset approximately two to four bp from the polyadenine-directed curving. The examination of the nucleotide sequence of Alu156 reveals the presence of several adenine di- and trinucleotides upstream from the three polyadenine runs. The junction bend model (Koo and Crothers, 1988; Koo et al., 1986; Wu and Crothers, 1984) for curvature predicts the 3' ends of the polyadenine groups to contribute more to the overall curvature of a molecule than the 5' ends of these regions. Several of the di- and trinucleotides (i.e.,
those nearest -130, -120, -110, -100, and -80) have 3' ends which are
spaced at 9-11 bp intervals and are offset from the 3' end of the
middle polyadenine region (-51) by an average of three bp. Adenine
di- and trinucleotides have been predicted to contribute 5-11 degrees
each to sequence curvature (Ulanovsky et al., 1986) whereas each
polyadenine region is predicted to contribute about 19 degrees of
curvature to the helix of the Alu156 promoter based on the curvature
values of A₅N₅ and A₅T₅ (Koo and Crothers 1984). Since the three
polyadenine regions appear to be in phase with one another they would
be predicted to additively contribute approximately 57 degrees of
essentially unidirectional curvature to the Alu 156 promoter. The
adenine di- and trinucleotides located further upstream would be
predicted to contribute less to the curvature of the molecule because
(1) each one yields less net curvature than an A₅N₅, A₅T₅, or A₅N₆
group and (2) the phasing between the 3' ends of the five adenine di-
and trinucleotides mentioned above is less precise than that observed
for the polyadenine regions. Taken together these observations imply
that the overall curvature from the adenine di- and trinucleotides
would be less than that calculated for the polyadenine-induced
curvature and would be offset approximately 70 to 100 degrees with
respect to the polyadenine-induced curvature.

Adenine di- and trinucleotides seem to impart a less stable,
(i.e. more flexible) curvature than that induced by runs of four or
more adenines as the fact that poly d(A)·polyd(T) cannot be bent into
a nucleosome structure (Simpson and Kunzler, 1979), however DNA
sequences containing phased adenine dinucleotides are easily bent into
nucleosome structures. An important difference in the flexibility of
DNA containing helically phased adenine dinucleotides vs DNA containing longer groups of adenines with the same phasing may involve the difference in the length of the non-curved DNA between the adenine groups of the two molecules. The major portion of the peaks seen in Figure 3 does not seem to be further stabilized by lowering the temperature from 23°C to 5°C. However the skewed right side of the peak represents a further reduction in electrophoretic mobility at 5°C (see +9, and +11 and +19 and +21 values). This suggests that the skewing is due to the presence of curvature which is offset by two to four bp and less stable at room temperature than the polyadenine-induced curve.

From Figure 1 it is also apparent that the only adenine runs downstream from the -35 region are the interrupted regions whose 3' ends are at -5 and +5 respectively. While these regions would located on the same side of the helix, they are located on the opposite side of the helix from the upstream polyadenine regions. The region ending at -5 is comprised in part by the -10 region of the promoter. The A + T rich -10 region has previously been proposed as a possible site of DNA curvature (Trifanov and Sussman, 1980; Trifanov, 1986). The phasing of this region of Alu156 with respect to the upstream polyadenine regions would be predicted to yield maximum retardation of electrophoretic mobility when rotated by 1/2, 1-1/2, and 2-1/2 turns on the helix, as seen for the +6, +17, and +25 values.

Role of curved DNA in transcription initiation. Two major models have been proposed to explain the role of curved upstream DNA in the activation of transcription. The curvature of the DNA upstream from the -35 region may allow it to wrap around the upstream end of the RNA
polymerase molecule, to proved additional contacts between enzyme and promoter (Bossi and Smith, 1984). This model would require that the direction of upstream curvature be oriented toward the side of the helix where the -35 region is located. This type of model could explain the rotation effect observed with the Alul56 insertion mutants. E. coli and B. subtilis promoters have been identified (Peschke et al., 1985; Plaskon and Wartell, 1987) which contain polyadenine regions around -45 (one helical turn from the -35 region). It has been suggested that these additional contacts might serve to increase the isomerization from the closed complex to a stable intermediate complex, in the following kinetic scheme:

$$R + P \xrightarrow{K_B} RP_C \xrightarrow{K_I} RP_i \xrightarrow{K_F} RP_O.$$

This has been identified as the rate limiting step in transcription initiation of the lambda PR promoter (Roe, et al., 1984, 1985) and lacUV5 promoter (Buc and McClure, 1985) at temperatures greater than 25°C.

A possible second role for the curved upstream regions involves the binding of additional RNA polymerase molecules. Increasing the local concentration of enzyme adjacent to a promoter via a "loading dock" effect has been proposed to primarily increase the initial binding of RNA polymerase to form a closed complex at the promoter (Plaskon and Wartell, 1987; Travers et al., 1983).

Promoters such as that for tyrT and for rRNA genes contain regions of curved DNA upstream from -35. However they also contain sequences resembling alternative binding sites for RNA polymerase. These consist of properly spaced -35 and -10 regions which generally show poor homology with the consensus sequences relative to the
primary promoter site. These secondary binding sites may be inverted with respect to the primary site. They have been shown to bind additional RNA polymerase molecules by in vitro protection experiments (Travers et al., 1983). Promoters in which the upstream curved regions and secondary binding sites were deleted showed decreased transcription from the tyrT promoter (Lamond and Travers, 1983).

We have detected a possible secondary binding site in Alul56 which would have -35 and -10 regions located at -89 and -66, respectively, in relation to the Alul56 start site (Figure 1). The secondary site is 63% homologous with the E. coli consensus sequence, as determined by the parameters of Mulligan et al. (1984). This is considerably lower than the 83% homology score determined for the Alul56 promoter site, moreover, we have been unable to detect runoff transcription products from this site in vitro. In a previous report, we demonstrated that the DNA upstream from -35 from another SP82 early gene promoter was able to effectively replace that of Alul56. This substituted upstream DNA also contained three polyadenine regions, but only extended to -95 and did not contain a secondary RNA polymerase binding site (McAllister and Achberger, 1988). This argues strongly against the effect of the Alul56 upstream regions being due to the binding of additional RNA polymerase molecules and supports a role for increased binding of the polymerase molecule at the promoter site. Probably the strongest argument against a "loading dock" model is the rotation orientation dependence of transcription stimulation. If the upstream, curved DNA was simply a binding site, it would function independent of orientation. Additionally, we found the largest differences in binding of RNA polymerase among mutant constructions
were at the lowest enzyme concentrations tested. At these concentrations, one would expect that a secondary site with a much lower homology score would not compete effectively with the promoter site found on each mutant. In addition, no evidence was found for the cooperative binding of two RNA polymerase molecules to the Alu156 promoter.

A modification of the first model would be one in which the upstream regions increase binding of a single polymerase molecule at the promoter site by providing additional contacts through DNA looping. This model would predict the large differences in binding seen for the wild type vs deletion mutants at low RNA polymerase concentrations. It may be that the additional curvature provided by the adenine di- and trinucleotides upstream from the polyadenine regions aids in the formation of a DNA loop. When the upstream DNA is rotated by 1/2 or 1-1/2 turns loop formation would be more difficult. The somewhat higher transcription rates and binding seen for the +6 and +17 promoters compared to mutants in which the upstream polyadenine regions have been deleted entirely (McAllister and Achberger, 1988) may reflect, that some degree of looping occurs even when the polyadenine regions are rotated opposite the -35 region on the DNA helix. Arguing against the DNA looping model is the fact that replacement of the Alu156 upstream region with one that extends only up to -95 resulted in levels of in vitro transcription, RNA polymerase binding, and CAT expression similar to those seen for Alu156.

The first model in which the DNA sequence within the curved polyadenine regions interacts directly with RNA polymerase at the promoter to provide additional contacts via a "wraparound" effect
cannot at this point be excluded. This model would also predict that rotation of the upstream regions to the opposite side of the helix from the original orientation would decrease promoter utilization by eliminating the additional contacts. It is unclear however, whether there is enough flexibility in the polyadenine synthetic insertion regions to allow them to bend so that the RNA polymerase can contact the polyadenine region in the +11 and +21 insertion mutants. The +11, +21, and +29 mutations would result in the linear displacement of these regions by approximately 3.6, 6.8, and 9.4 nm.

The final determination of the mechanism responsible for the rotational dependence of the orientation of the upstream curvature to the promoter proper will require further study. We are presently constructing mutant promoters with oligonucleotide insertions of less than 6 bp as well as mutants with a greater than 29 bp of inserted DNA. We are also analyzing Alu156 derivatives which have the DNA upstream from the polyadenine regions deleted. Studies similar to those reported here with these mutant promoters should enable us to ascertain whether a "looping" or a "wraparound" mechanism is responsible for the stimulation of transcription by these curved regions. Kinetic studies, such as temperature shift experiments (Buc and McClure, 1985; Roe et al., 1984, 1985), using these new mutants, as well as, those described in this report and in a previous one (McAllister and Achberger, 1988) should enable us to identify what step(s) of initiation are most affected by the curved DNA.
Fig. 1. Nucleotide sequence of the Alul56 promoter. Bases representing the +1 transcription start site, the -35 region, and the -10 region are in bold type. The runs of adenines in the nucleotide sequence immediately upstream of the -35 region are underlined. Regions of regularly spaced adenine di- and trinucleotides are marked with a line over the sequence.
GAATTC

CTTCAAGAAGATATCCAACAGGCAAAGAGGGAATACTCTCATCAGAAACAACCTCTG

CTAAAAATTCCTGAAAAATTTTGCAAAAAGTTGTTGACTTTCTCTAGGAGGTTGGCATAATAATTTAAC

AACAGCAGGACCGCTAGGACGGATCCGGGGAATTG
Fig. 2. **Nucleotide sequence of the DNA immediately upstream from the -35 region of the Alu156 derived mutant promoters.** The numbering of the nucleotide sequence corresponds to the +1 transcription start site of the Alu156 promoter presented in Fig. 1. The DNA insertions for each mutant are underlined, and the runs of adenines are in bold type. The rotational displacement based on B-form DNA is given in helical turns for each.
Rotational Displacement

| Alu156 | CATCCAGAACAACTCTGCTAAAAATTCCTGAAAAATTTTGCAAAAAAGTTGTTCCGATTTGACT | 0.0 |
| +6     | GAACAAACCTCTGCTAAAAATTCCTGAAAAATTTTGCAAAAAAGTTGTTCCGATTTGACT | 0.57 |
| +9     | CAAACCTCTGCTAAAAATTCCTGAAAAATTTTGCAAAAAAGTTGTTCCGACGATTTGACT | 0.86 |
| +11    | ACCTCTGCTAAAAATTCCTGAAAAATTTTGCAAAAAAGTTGTTCCGGATCGATTTGACT | 1.05 |
| +13    | CTCGCTAAAAATTCCTGAAAAATTTTGCAAAAAAGTTGTTCCGATCGATTTGACT | 1.24 |
| +15    | CTGCTAAAAATTCCTGAAAAATTTTGCAAAAAAGTTGTTCCGCGATCGATTTGACT | 1.43 |
| +17    | GCTAAAAATTCCTGAAAAATTTTGCAAAAAAGTTGTTCCCGGATCGACGATTTGACT | 1.62 |
| +19    | TAAAAATTCCTGAAAAATTTTGCAAAAAAGTTGTTCCGATCGACGATTTGACT | 1.81 |
| +21    | AAATTTCTGAAAAATTTTGCAAAAAAGTTGTTCCGATCGACGATTTGACT | 2.0 |
| +25    | TCCTGAAAAATTTTGCAAAAAAGTTGTTCCGCGGATCGATCGACGATTTGACT | 2.38 |
| +29    | GAAAAATTTTGCAAAAAAGTTGTTCCCGGATCGTGGATCGTGGATCGACGATTTGACT | 2.76 |
Fig. J. Effect of temperature on the electrophoretic mobility of DNA fragments containing the Alu156 promoter or insertion mutant promoters. The ratio of the apparent molecular weight based on electrophoretic mobility to actual molecular weight determined from the nucleotide sequence is presented as a function of the length of the oligonucleotide insertion for the Alu156 promoter and insertion mutants. The apparent molecular weight of promoter-containing DNA fragments was determined using pBR322-MspI and pBR322-HaeIII molecular weight markers as described under "Experimental Procedure". The temperatures used during electrophoresis were 5°C (●), 23°C (○), and 65°C (△).
Fig. 4. Effect of oligonucleotide insertions on promoter utilization in *B. subtilis*. Panel A: Promoter-specific chloramphenicol acetyltransferase (CAT) specific activity as a function of the insertion length. CAT specific activity, μmol chloramphenicol acetylated per minute per milligram protein, for each promoter construction was determined from three independent transformants containing the correct pPL703-promoter transcriptional fusions. Panel B: Nitrocellulose membrane retention of RNA polymerase bound promoter-containing DNA fragments expressed as the fraction of input DNA bound normalized to the binding observed for the internal control, the Alul56 promoter containing fragment. All binding reactions were performed at an RNA polymerase/DNA weight ratio of 1.9 using the conditions described under "Experimental Procedure". Panel C: The effect of oligonucleotide insertion length on RNA synthesis measured by the incorporation of 3H-UTP in to acid precipitable material as described under "Experimental Procedure". Purified RNA polymerase was used to transcribe either supercoiled (Δ) or linear (▴) pUC8 derivatives containing the individual promoter constructions at an enzyme/DNA weight ratio of 0.9.
Fig. 5. Relative affinity of B. subtilis RNA polymerase for DNA fragments containing the Alu156 promoter or insertion mutant promoters. The competition binding assay measured the fraction of promoter containing DNA retained on a nitrocellulose membrane filter as a function of RNA polymerase concentration. At each RNA polymerase concentration, the reaction contained equal amounts of DNA fragments containing the Alu156 promoter (○), the +6 mutant (●), the +11 mutant (△), and the +15 mutant (▲). RNA polymerase binding was quantitated as described under "Experimental Procedure".
CHAPTER III

Altered Utilization of Lambda Phage $P_L$ and $P_R$ Promoters in *B. subtilis* After the Addition of Curved DNA Sequences.
INTRODUCTION

*B. subtilis* promoters have the same conserved hexanucleotide sequences at -10 and -35 as the consensus sequence derived for promoters from *E. coli* (Lee *et al.*, 1980b; Moran *et al.*, 1982; Murray and Rabinowitz, 1982; Graves and Rabinowitz, 1986). Although many *B. subtilis* promoters are efficiently utilized by *E. coli* sigma-70 containing RNA polymerase, most *E. coli* promoters do not function well in *B. subtilis* (Lee *et al.*, 1980a; Shorenstein and Losick, 1973; Wiggs *et al.*, 1979; Achberger and Whiteley, 1981). This suggests that DNA sequences other than the -10 and -35 regions are important for the utilization of promoters by *B. subtilis* sigma-43 containing RNA polymerase. These sequences were proposed to include a polyadenine region at -45 and a conserved pentanucleotide sequence located from -18 to -14 which is termed the -16 region (Graves and Rabinowitz, 1986; Peschke *et al.*, 1985). It was previously demonstrated that the presence of three polyadenine tracts which are located at -45, -55, and -65 relative to the transcriptional start site are required for efficient in vivo and in vitro transcription from a *B. subtilis* phage SP82 promoter recognized by the major *B. subtilis* RNA polymerase. These upstream sequences increased binding of *B. subtilis* sigma-43 containing RNA polymerase in vitro (McAllister and Achberger, 1988).

In this report, the effect of these upstream polyadenine-containing regions on promoter utilization in *B. subtilis* and *E. coli* was examined. In vivo transcription was compared among wild type, hybrid, and deletion mutants of two SP82 phage early gene promoters.
The affinity of RNA polymerase for *E. coli* phage lambda promoters with and without the polyadenine-containing upstream DNA from SP82 promoters was examined.
EXPERIMENTAL PROCEDURE

Construction of mutant promoters from phage lambda promoters $P_R$ and $P_L$. The phage lambda promoters $P_R$ and $P_L$ were both isolated from restriction fragments of the plasmid vector pGW7 (Deutch et al., 1982). This expression vector is a pBR322 derivative into which a 4.0 kilobase (kb) fragment containing the lambda phage control region was inserted. The promoters $P_R$ and $P_L$ were excised from this region of the vector as a 194 bp AluI fragment and a 352 bp HaeIII fragment, respectively. Each purified fragment was blunt-end ligated into the HincII site of pUC8, and constructions were screened for those with the promoter oriented in the same direction as the lac promoter in the vector. The nucleotide sequence of each lambda promoter (Daniels et al., 1983) shows that each contains a HincII recognition site which permitted these promoters to be cleaved at -33, within the -35 region (Figure 1). The promoter DNA upstream from -33 was deleted by digestion with EcoRI and HincII and subsequent purification of the linear vector as described below when general techniques. The isolation and cloning of the B. subtilis promoters Alu156 and Ball29 was described (McAllister and Achberger, 1988). These promoters also contain a HincII site at -33. The DNA upstream from this site for each of these promoters was purified as an EcoRI, HincII restriction fragment by polyacrylamide gel electrophoresis as described previously (McAllister and Achberger, 1988). These fragments were then substituted for the $P_R$ and $P_L$ upstream fragments by ligating them into the sites where the original upstream regions were deleted. This scheme was used to create four hybrid promoters which contained B.
*subtilis* phage SP82 derived promoter sequence upstream from -33 and phage lambda derived promoter sequence downstream from -34 (Figure 1).

Each of the two wild type and the four hybrid promoters could be cleaved from the vector with EcoRI and PstI, EcoRI and HindIII, BamHI and PstI, or BamHI and HindIII. The sizes for EcoRI, PstI fragments of each of the promoters are as follows: PL 374 bp, Alu156-PL 273 bp, Ball29-PL 212 bp, Pr 216 bp, Alu156-Pr 234 bp, Ball29-Pr 173 bp. Mutant promoters were confirmed by a combination of restriction digest analysis and nucleotide sequencing (Sanger et al., 1977).

In vivo chloramphenicol acetyltransferase (CAT) expression. Each of the promoter constructs were excised from the vector with EcoRI and PstI, purified using polyacrylamide gel electrophoresis, and ligated with vector pPL703 (Duvall et al., 1983) that had been digested with the same enzymes. Transcriptional fusions were created between each promoter construction described above and the cat gene of pPL703. These recombinant plasmids were transformed into *B. subtilis* 1A510 (Bacillus Genetic Stock Center, The Ohio State University; Ostroff and Pene, 1984) protoplasts by a modification (McAllister and Achberger, 1988) of the method of Chang and Cohen (1979). Selection and screening of transformants, CAT assays (Shaw, 1975) and protein concentration determinations (Bradford, 1976) were performed as previously described (McAllister and Achberger, 1988).

In vivo β-galactosidase expression. The isolation and cloning of Alu156 and Ball29 promoters from the *B. subtilis* phage SP82 have previously, as have the construction of the hybrid and -34 deletion mutants of each promoter (McAllister and Achberger, 1988). Each of these promoters was cleaved from an M13 mp7 construction as a BamHI
fragment. The 5' termini of these fragments were made flush with E. coli DNA polymerase I large fragment and dATP, TTP, dCTP, and dGTP. These fragments were purified by polyacrylamide gel electrophoresis and each was ligated into the promoter cloning vector pMC306 (Casadaban and Cohen, 1980) and transformed into E. coli MC1000 competent cells (Maniatis et al., 1982). Transformants were selected and screened on MacConkey agar (Difco Laboratories, Detroit, MI) plates containing 40 µg/ml ampicillin. Colonies which are red indicate insertions of a promoter in the correct orientation to give transcription of the lacZ reporter gene. Positive colonies were subcultured into L broth (Maniatis et al., 1982) with 40 µg/ml ampicillin and grown to an O.D. of 0.6, and 3 ml of culture was sedimented in a microfuge and suspended in 0.4 ml of Z buffer (Miller, 1972). These cells were subjected to 6 x 5 seconds of sonic disruption at 0°C, and the cell-free extract was assayed for β-galactosidase activity (Miller, 1972).

RNA polymerase isolation. B. subtilis Ea438 RNA polymerase was isolated (Achberger and Whiteley, 1981; Spiegelman et al., 1978) with modifications described previously (McAllister and Achberger, 1988). E. coli E d70 RNA polymerase was isolated using a similar procedure (Spiegelman et al., 1978) with some modifications (Achberger and Whiteley, 1980).

RNA polymerase binding assays. The B. subtilis or E. coli RNA polymerase retention of promoter-containing DNA fragments on nitrocellulose membrane filters was based on the method of Jones et al. (1977) and modifications have been described previously (McAllister and Achberger, 1988). The competition binding assay
measured the filter retention of various promoter-containing DNA fragments relative to one another as a function of RNA polymerase concentration.

The EcoRI, PstI fragments containing each of the six lambda-derived promoters differ enough in molecular weight to be easily resolved on a 6% polyacrylamide gel with the exception of Pr at 216 bp and Ball29-PL at 212 bp. To solve this problem, the size of the Pr promoter fragment was increased to 224 bp by excising it from the vector with EcoRI and HindIII, and the size of the Ball29-PL fragment was decreased to 203 bp by digesting it with BamHI and PstI. In each 0.4 ml competition binding assay, 1.5 ug of digested plasmid was added for each promoter tested. The pUC8 vector contains no promoters which are efficiently bound by B. subtilis £043. Although E. coli £070 exhibits greater binding to the vector DNA, this level is still less than 20% of that seen for any of the lambda-derived promoter constructions. We therefore included the vector DNA in the binding reactions to minimize non-specific binding, such as end binding (Melancon et al., 1983), to the test fragments.

Polyacrylamide gel electrophoresis. Electrophoresis to separate small promoter containing DNA fragments for densitometric quantitation of filter binding studies employed 6% (60:1 monomer to bis ratio) polyacrylamide gels. Electrophoresis conditions were 7 volts/cm for 5 hr at room temperature. For fragment mobility determinations, 10% (60:1 monomer to bis ratio) polyacrylamide gels were used. Electrophoresis conditions for fragment mobility determinations were made at 6.5 volts/cm (also limited to 24 milliamps) for 11 hr at 23 C. All gels utilized a TBE buffer system (89 mM Tris, 89 mM boric acid,
2.5 mM disodium EDTA, pH 8.3). For fragment mobility determinations, a peristaltic pump was used to recycle running buffer between the upper and lower reservoirs at a rate of 10 ml/min. For routine screening of constructions and for preparative isolation of promoter containing DNA fragments, either 6% or 10% gels were employed at 10 volts/cm (and limited to 25 milliamps).

**General techniques.** Transfection of E. coli JM101 and transformation of E. coli JM83 was accomplished with CaCl2 treated cells (Maniatis et al., 1982). Plasmid DNA and M13 replicative form was isolated using the alkaline lysis method (Birnboim and Doly, 1979) and CsCl gradient density gradient centrifugation in the presence of ethidium bromide. Manufacturers recommendations were followed in the use of restriction enzymes and DNA modifying enzymes. Following electrophoresis and staining with ethidium bromide, DNA fragments to be purified were excised from polyacrylamide gels with a razor blade, minced, and eluted overnight at 37°C in TBE buffer. The DNA was concentrated and further purified using DE52 (Whatman) chromatography and ethanol precipitation. CsCl purified vector DNA which was digested with more than one restriction enzyme was electrophoresed on a 0.6% agarose gel to separate it from the excised linker, visualized by U.V. shadowing over a fluorescent screen, excised with a razor blade, and electroeluted from the gel slice in a dialysis bag. The DNA-containing eluent was then phenol extracted, ether extracted, and ethanol precipitated.
RESULTS

To determine the ability of the polyadenine regions upstream from the
-35 region of the Alu156 and Ball29 promoters to stimulate
transcription from heterologous promoters, we constructed a series of
four mutant promoters in vitro. The DNA upstream from -35 in Alu156
and Ball29 promoters was substituted for the analogous region in the
phage lambda promoters Pr and Pl. The nucleotide sequence (Daniels et
al., 1983) of the Pr and Pl fragments is presented in Figure 1.
Neither promoter has polyadenine runs immediately upstream of the -35
region. The homologies with the E. coli consensus sequence are 58%
and 57%, respectively, for Pl and Pr using the parameters of Mulligan
et al. (1984). The -35 regions of Pr and Pl differ from each other at
-30 but are identical to the corresponding regions of Alu156 and
Ball29, respectively. The lambda promoters both contain a seventeen
base pair spacer region between the -10 and -35 regions. Fusion of
the upstream DNA from promoters Alu156 and Ball29 to the lambda phage
Pr and Pl promoters conserved the orientation between the curved
upstream DNA and the promoter observed for the B. subtilis promoters.

In vivo expression from promoter constructions. The promoter
cloning vector pPL703 (Duvall et al., 1983) was used to establish
transcriptional fusions between each of the lambda-derived promoters
and the chloramphenicol acetyltransferase (CAT) gene carried on the
plasmid. The levels of CAT specific activity measured for the
different promoter constructions are presented in Table 1. The lambda
Pl promoter yielded the lowest CAT specific activity. Replacing the
Pl upstream region with that of Alu156 or Ball29 increased CAT
expression at least four fold. The lambda Pr promoter expressed about
10 times the CAT specific activity of PL in B. subtilis. However this level is still three-fold less than that seen for Ball29 and six-fold less than the level seen for Alul56. When the upstream region from Ball29 was used to replace that of PR the resulting Ball29-PR hybrid exhibited more than a five-fold increase in CAT specific activity. The levels of CAT expression for these three hybrid promoters indicate a stimulatory effect of the phased polyadenine regions upstream from -35 on transcription in B. subtilis. The Alul56-PR hybrid however exhibited a five-fold decrease in CAT specific activity compared to the PR promoter. This was surprising, since the same upstream sequence had stimulated expression from the lambda PL promoter. To resolve this discrepancy and to better understand the differences between the interactions of promoters with B. subtilis Eσ43Δ and E. coli Eσ70 RNA polymerases, the in vitro binding of these enzymes to each of the six promoter constructions was examined.

In vitro RNA polymerase binding assays. To test if the levels of in vivo promoter activity reflected changes in affinity for RNA polymerase, competition binding assays were performed in which equal molar amounts of each of the six promoter constructions competed for substituting concentrations of either E. coli Eσ70 or B. subtilis Eσ43Δ RNA polymerase. The results of these assays are presented in Figures 2 and 3. The PL derived promoters all appeared to bind B. subtilis RNA polymerase better than the PR derivatives (Figure 2). These results are opposite those we see for in vivo CAT expression where the three PL derivatives gave lower CAT specific activities than all but the Alul56-PR hybrid promoter. Within the PR and PL derivative groups the results are more difficult to interpret.
Ball29-PL binds B. subtilis RNA polymerase much better than any of the other promoters. The Alul56-PL hybrid, which produced a level of CAT specific activity equal to Ball29-PL, was bound by RNA polymerase much less efficiently (i.e., below the level of binding seen for the wild type PL promoter). The P_R derivative promoters demonstrated an inverse correlation between RNA polymerase binding in vitro, and in vivo CAT expression.

When the binding of the promoter fragments by E. coli RNA polymerase was examined no significant differences were observed among the six constructions (Figure 3). This is consistent with reports that E. coli RNA polymerase does not discriminate between promoter sequences as stringently as does B. subtilis RNA polymerase (Achberger and Whiteley, 1980; Pawlyk, 1986; Peschke et al., 1985). In general, at lower enzyme concentrations all six promoters were bound much more efficiently by E. coli RNA polymerase than by B. subtilis RNA polymerase.

Since gene expression from B. subtilis promoters is highly affected by the upstream polyadenine tracts, it was of interest to test if these promoters would demonstrate the same dependency when utilized in E. coli in vivo. To examine this, we created transcriptional fusions between the previously described promoters (i.e. the wild type, the hybrid, and the -34 deletion constructions of the Ball29 and Alul56 promoters, McAllister and Achberger, 1988) and a lacZ reporter gene on the E. coli promoter cloning vector pMC306 (Casadaban and Cohen, 1980). The level of β-galactosidase specific activity measured for each of the constructions in E. coli MC1000 is presented in Table 2. These activities are displayed along with the
levels of in vivo CAT expression measured for the same promoters in B. subtilis 1A510 that were previously determined (McAllister and Achberger, 1988). It is important to note that the absolute values for expression from each promoter in E. coli vs B. subtilis cannot be directly compared, however the trends between different derivatives of the same promoter can be compared. In B. subtilis, both of the hybrid promoters displayed CAT activities which were at least one-third lower than the wild type promoters. This presumably reflects fine tuning of the upstream sequence and the sequence of the downstream promoter proper to give efficient transcription from each wild type promoter. The Alul56 promoter which does not contain the consensus -10 and -35 regions seems to depend on the upstream regions for stimulation of transcription. This may be the reason that the upstream region of Ball29, which has less precisely phased polyadenine tracts than those of the Alul56 upstream region (Fig. 1), does not allow as high a level of transcription from the Alul56 hybrid construction as we observed for the wild type promoter. The deletion of the upstream sequence and alteration of the -35 region result in almost complete loss of promoter function in the Alul56 -34 deletion.

Ball29 which contains the consensus -10 and -35 regions, does not depend on the upstream polyadenine tracts for efficient transcription and the activity of the Ball29 hybrid is adversely affected by the Alul56 upstream regions, which increase its affinity for RNA polymerase. Thus, the loss of the upstream polyadenine regions and alteration of the -35 region do not seem to adversely affect transcription from the Ball29 -34 deletion construction.
The *E. coli* in vivo expression levels show much less variability due to the upstream polyadenine tracts. The less precise phasing of the Ball29 polyadenine tracts only reduces the activity of the Alu156 hybrid 7% compared to the wild type. Moreover, the Ball29 hybrid actually shows a 7% increase over the expression level observed for Ball29. Thus as in the *in vitro* studies with the lambda-derived promoters, *E. coli* RNA polymerase appears to respond very little to the presence or absence of the upstream polyadenine tracts. Since this enzyme appears to interact primarily with the promoter sequences downstream from -35, it is not surprising that expression is severely reduced for the Alu156 -34 deletion since the -35 region has been altered. However the expression level in *E. coli* is still greater than the level seen in *B. subtilis* where transcription is more dependent on not only the -35 region sequence but also the upstream regions. With the Ball29 -34 deletion the loss is only 40%, perhaps because the *E. coli* RNA polymerase is only responding to a two nucleotide change in a consensus -35 region and not to the concomitant loss of the upstream polyadenine regions. The decrease in activity from the Ball29 -34 deletion might be expected to be greater if the -10 region and unaltered portion of the -35 region were not identical to the *E. coli* consensus promoter sequence.

**Electrophoretic mobilities of promoter-containing DNA fragments.** The lambda-derived promoter fragments migrated on polyacrylamide gels at rates consistent with DNA molecules 10-14% larger than their actual size (Table I). PL seems to contain more polyadenine tracts (Figure 4; Daniels et al., 1983) than does PR and consequently has a more aberrant electrophoretic mobility. It is interesting that neither
molecule exhibits the degree of altered mobility observed for the Alu156 and Ball29 promoters (McAllister and Achberger, 1988). This can be attributed to less precise phasing of the polyadenine regions in the upstream region of the lambda promoters.

The upstream regions of the Ball29 and Alu156 promoters affected the mobility of DNA fragments with the two lambda promoters differently. In general, the replacement of the upstream DNA of the lambda \( P_L \) promoter with that of the \( B. \ subtilis \) promoters did not significantly change the mobility. In contrast, the analogous exchange to create the \( P_R \) hybrid promoters lead to a decrease in the electrophoretic mobility. This suggests that the curved DNA fragments from the Alu156 and Ball29 promoters altered the DNA structure of the \( P_R \) hybrid promoters differently than they did the \( P_L \) hybrid promoters.
DISCUSSION

The phage lambda promoters Pr and Pl are strong E. coli promoters which have been used in a number of recombinant plasmid vectors, such as pGW7 (Deutch et al., 1982), designed for high expression of the products of molecularly cloned genes. The utilization of the lambda Pl promoter by E. coli RNA polymerase was shown to be greatly influenced by the presence of the polyadenine tracts upstream from -69 (Horn and Wells, 1981). Loss of this upstream region was associated with a two-fold decrease in initiation and in decreased stability of preformed RNA polymerase:promoter complexes when challenged with heparin. In addition, the deleted promoter required a higher temperature to form stable complexes than did the wild type promoter.

Recently, the activity of the E. coli trp promoter was modulated by the upstream polyadenine tracts from the lambda Pl promoter. The trp promoter contains two polyadenine containing tracts which extend from -43 to -56 and from -82 to -93 on the promoter. Deletion of both of these tracts was accompanied by a seven-fold reduction in transcription. Replacement of the trp promoter DNA upstream from the -35 region with a portion of the lambda upstream region extending to -100 resulted in a 21-fold increase in transcription above that seen for the deleted trp promoter, or a three-fold increase over the level seen for the wild type trp promoter.

When the ability of the Ball29 and Alul56 upstream regions to modulate promoter utilization in E. coli was tested, the polyadenine tracts exhibited a much less pronounced effect than in B. subtilis. This may relate to the fact that the polyadenine region at -45 is a more highly conserved feature of B. subtilis promoters than those of
E. coli (Graves and Rabinowitz, 1986). E. coli E_70 RNA polymerase did not discriminate between the lambda wild-type and hybrid promoters in vitro, supporting the idea that regions other than the -35 and -10 regions are less important for promoter function with E. coli E_70 RNA polymerase than with B. subtilis E_43δ RNA polymerase.

In general, the in vitro binding of B. subtilis E_43δ RNA polymerase by the lambda derived promoters was not correlated to CAT activity. This was opposite the effect we had observed previously for the Alul56 promoter (McAllister and Achberger, 1988) in which the presence of the upstream regions was associated with increased in vivo expression and in vitro binding of B. subtilis E_43δ RNA polymerase. The decrease in CAT activity observed for the Alul56-PR promoter does not appear to be similar to the effect of the upstream regions on the Ball29 promoter which we reported previously (McAllister and Achberger, 1988). The lambda promoters PR and PL have -35 regions identical to Alul56 and Ball29 respectively, and all four promoters have a 17 bp spacer sequence. These sequence similarities permitted the heterologous upstream DNAs to be spliced onto the lambda promoters in the same orientation as was found in the Alul56 and Ball29 promoters. It is interesting to note however that the nucleotide sequence of PR and PL differ from that of Ball29 and Alul56 in two regions located downstream from the -35 region which are important in interactions with B. subtilis E_43δ RNA polymerase (i.e., the -16 region and the -10 region). B. subtilis promoters contain a highly conserved sequence from -18 to -14, termed the -16 region (Graves and Rabinowitz, 1986). The two most common sequences at the -16 region of B. subtilis promoters recognized by the major RNA
polymerase are 5' CCATG 3' or 5' CTGTG 3'. The thymine and guanine at -15 and -14 respectively, are highly conserved in these promoters. The -16 region of \( P_R \) and \( P_L \) contains only the guanines at -18 and -14 which match the \( B. \ subtilis \) consensus sequence. \( P_R \) and \( P_L \) also have relatively poor homology in the -10 region with the consensus sequence derived for \( E. \ coli \) and \( B. \ subtilis \) (i.e., 5' TATAAT 3'). In particular, both promoters have a guanine at -12 which is extremely rare for \( B. \ subtilis \) promoters.

Promoter function appears to be modulated by a complex interplay of the effects of different regions of sequence which either interact directly with the RNA polymerase, or alter the orientation of sequences that interact with the enzyme (Auble et al., 1986). It should be noted that the upstream curved regions from Alul56 and Ball29 are not identical, that is, these two regions affected the utilization of SP82 promoters differently in \( B. \ subtilis \) (McAllister and Achberger, 1988). Subtle changes in nucleotide sequence of the lambda \( P_R \) and \( P_L \) promoters may require a precise orientation of the curved DNA. This may be especially true when DNA structuring take place immediately next to a protein binding site (Johnson and Simon, 1985; Johnson et al., 1986). It is not unexpected that each of the upstream DNAs from Alul56 and Ball29 did not create a productive combination (i.e., functional promoter) with both of the lambda promoters.

The sequence differences seen in the lambda promoters and the SP82 phage promoters may result in the existence of different rate limiting steps in transcriptional initiation from the lambda derived promoters compared to Alul56 and Ball29. The upstream polyadenine
regions were able to increase in vivo expression in three out of four lambda hybrid promoters. It appears that the upstream polyadenine regions may affect more than one step in the process of transcriptional initiation. In this regard, these regions may be similar to supercoiling which has been shown to affect several steps in transcription initiation (Buc and McClure, 1985). The affected step must most likely be "rate limiting" to see a stimulation of transcription by these regions. It is not yet known what step is rate limiting for initiation from P_R and P_L, however the results of this report suggest that it is probably not RNA polymerase binding. Further investigation will be required to determine other step(s) in the initiation of transcription from the lambda promoters which may be affected by polyadenine tracts upstream from the -35 region. Such further studies may include the determination of 1) initiatable complexes formed during binding assays and 2) stability of preformed RNA polymerase:promoter complexes among the various lambda-derived promoters.
Table I

Apparent molecular weights and in vivo utilization of lambda wild type and hybrid promoters.

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Fragment Length&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Apparent MW&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Actual MW</th>
<th>CAT Specific Activity&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL</td>
<td>374</td>
<td>1.14</td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>Alu156-PL</td>
<td>273</td>
<td>1.11</td>
<td></td>
<td>0.18</td>
</tr>
<tr>
<td>Ball29-PL</td>
<td>212</td>
<td>1.07</td>
<td></td>
<td>0.17</td>
</tr>
<tr>
<td>PR</td>
<td>216</td>
<td>1.10</td>
<td></td>
<td>0.42</td>
</tr>
<tr>
<td>Alu156-PR</td>
<td>234</td>
<td>1.20</td>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td>Ball29-PR</td>
<td>173</td>
<td>1.20</td>
<td></td>
<td>2.27</td>
</tr>
</tbody>
</table>

<sup>a</sup>Length in base pairs determined from the nucleotide sequence.

<sup>b</sup>Apparent weight determined by electrophoretic mobility using pBR322 digested with either MspI or HaeIII as molecular weight markers as described under Experimental Procedure.

<sup>c</sup>Specific activity of chloramphenicol acetyltransferase expressed as micromoles of chloramphenicol acetylated per minute per milligram protein at 25°C.
Table II
In vivo utilization of Alul56 and Ball29 derived promoters in B. subtilis and E. coli

<table>
<thead>
<tr>
<th>B. subtilis</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT specific activity&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Percent of original promoter activity&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Alul56</td>
<td>2.6</td>
</tr>
<tr>
<td>Alul56 hybrid</td>
<td>1.7</td>
</tr>
<tr>
<td>Alul56 -34 deletion</td>
<td>0.1</td>
</tr>
<tr>
<td>Ball29</td>
<td>1.3</td>
</tr>
<tr>
<td>Ball29 hybrid</td>
<td>0.5</td>
</tr>
<tr>
<td>Ball29 -34 deletion</td>
<td>1.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Specific activity of chloramphenicol acetyltransferase expressed as micromoles chloramphenicol acetylated per minute per milligram protein at 25°C.

<sup>b</sup>The original promoters are designated Alul56 and Ball29.

<sup>c</sup>β-galactosidase specific activity expressed as nanomoles o-nitrophenol produced per minute per milligram protein at 25°C.
Fig. 1. **Nucleotide sequence of the Lambda P_L and P_R promoters.** The +1 transcription start site, the -10 region and the -35 region for the lambda P_L and P_R promoters are in bold type. The HincII restriction site used to construct the hybrid promoters with the upstream DNA from the Alul56 and Ball29 promoters is labeled. The nucleotide sequence of the upstream DNA from Alul56 and Ball29 used to make the hybrid promoters is listed and the runs of adenines contained therein are underlined.
Fig. 2. Relative affinity of B. subtilis RNA polymerase for the lambda $P_L$ and $P_R$ wild type and hybrid promoters. The competition binding assay measured the fraction of promoter DNA retained on a nitrocellulose membrane filter as a function of RNA polymerase concentration. At each enzyme concentration, the reaction contained equal molar amounts of DNA fragment containing the lambda $P_L$ promoter ($\bigcirc$), the lambda $P_R$ promoter ($\blacklozenge$), and the hybrid promoters, Alu156-$P_L$ ($\triangle$), Alu156-$P_R$ ($\triangleleft$), Ball29-$P_L$ (□), and Ball29-$P_R$ (■). RNA polymerase binding was quantified as described under "Experimental Procedure".
Fraction of DNA Bound
Fig. 3. Relative affinity of E. coli RNA polymerase for the lambda Pl and Pr wild type and hybrid promoters. The competition binding assay measured the fraction of promoter DNA retained on a nitrocellulose membrane filter as a function of E. coli RNA polymerase concentration. At each enzyme concentration, the reaction contained equal molar amounts of DNA fragments containing the lambda Pl promoter (O), the lambda Pr promoter (●), and the hybrid promoters, Alul56-Pl (△), Alu156-Pr (▲), Bal129-Pl (□), and Bal129-Pr (■). Lines connecting the data points are included for the two wild-type promoters. RNA polymerase binding was quantified as described under "Experimental Procedure".
Fig. 4. Nucleotide sequence of the upstream region of the lambda Pr promoter. The +1 transcription start site, -10 region, and -35 region are in bold type. Runs of adenines upstream of the -35 region are underlined.
CONCLUDING REMARKS

Several important findings were made during the course of this research. The polyadenine-containing upstream regions of Ball29 and Alu156 were found to increase the affinity of each promoter for RNA polymerase. The Ball29 promoter proper did not appear to be limited at the level of RNA polymerase binding. Therefore, the presence of the upstream regions resulted in decreased in vivo expression, probably due to decreased clearance of RNA polymerase from the promoter upon initiation. The Alu156 promoter appeared to be limited in RNA polymerase binding and was therefore dependent on the upstream regions for in vivo expression, in vitro transcription and in vitro binding of purified B. subtilis Ecoli RNA polymerase.

The 10-11 bp phasing of the polyadenine tracts conferred curvature to this DNA. The upstream sequence from Alu156, in which these tracts were more precisely phased, appeared to allow greater binding of RNA polymerase than did the corresponding region from Ball29. Electrophoretic mobilities of promoter containing fragments also indicated a second region of curvature downstream from -35.

Oligonucleotide insertion mutagenesis of the Alu156 promoter demonstrated that the rotational orientation of the upstream curvature relative to the promoter proper is more important to promoter function than the linear distance between these two regions. Maximal in vivo expression, in vitro transcription, and in vitro binding of RNA polymerase were observed when the upstream regions were rotated by one or two helical turns to restore the rotational orientation observed for the wild type promoter.
Electrophoretic mobilities of the mutant promoters suggest that in the Alul56 wild type promoter, the polyadenine-induced curve upstream from -35 directionally opposes the curve downstream from this point, conferring an S shape to the promoter. There appears to be a third region of curvature which is not precisely in phase with the polyadenine-induced curve. This region most likely consists of a group of adenine di- and trinucleotides which are further upstream from the polyadenine tracts and offset by two to four nucleotides with respect to their phasing on the DNA helix.

In vitro transcription from Alul56 promoters in which the upstream region had been deleted or rotated to the opposite side of the DNA helix could be partially restored if the DNA templates were supercoiled, suggesting that these regions may serve a function similar to supercoiling in the unwinding of these promoters during initiation. If the Alul56 and Ball29 promoters of the B. subtilis phage SP82 are transcribed prior to the circularization and supercoiling of the SP82 genome during the infection process, the upstream regions could be very significant.

The upstream polyadenine-containing sequence from Ball29 and Alul56 were able to dramatically alter the utilization of the lambda Pr and Pl promoters in B. subtilis. These lambda phage promoters are quite different from the two SP82 phage promoters in the -16 and -10 regions. Therefore, it appears that the upstream sequences are able to modulate transcription even in the absence of consensus sequence at other conserved regions. This implies that these sequences are important in the function of all promoters recognized by the major B. subtilis RNA polymerase.
Our findings are consistent with two models for the stimulation of transcription by these cis-acting curved sequences. The polyadenine tracts may interact directly with the RNA polymerase bound at the promoter to provide additional RNA polymerase:promoter contacts via a curvature-induced DNA "wraparound" mechanism. Alternatively, the curvature may allow the formation of a DNA loop so that regions upstream from the polyadenine tracts may provide these additional interactions with the RNA polymerase.

Neither the wraparound model nor the formation of DNA loops without the action of ancillary proteins have been previously demonstrated as a mechanism for the stimulation of transcription. Besides describing a novel mechanism for the stimulation of transcription initiation, this research should have practical applications. *Bacillus* species are used industrially for the overproduction of the products of cloned genes. It is easier to purify such products when produced by *Bacillus* sp. since the products can be encoded so that they are excreted from the cell into the growth medium. When these products are produced in *E. coli* they are contaminated with endotoxic material when the cells are disrupted to obtain the product. The characterization of the stimulation of transcription by properly aligned curved DNA will aid in the construction of more efficient expression vectors for the production of protein products in *Bacillus* sp.

Further work remains to be done to completely elucidate the role of curved DNA upstream from *B. subtilis* promoters. The use of oligonucleotide insertions longer than 29 bp and less than 6 bp, as well as the construction of curved regions which contain no DNA
sequence upstream from the polyadenine tracts will allow us to determine whether the wraparound or the DNA looping mechanism is responsible for the effects exerted by these upstream regions. It will also be interesting to see if all three polyadenine regions are necessary for optimal stimulation of transcription or if more than three of these regions further stimulate transcription from these promoters.

Synthetic oligonucleotides will be used to construct the upstream regions of these promoter mutants which will contain more than or less than three polyadenine tracts. The use of small chemical probes to determine the interaction of RNA polymerase with specific nucleotides in the sequence of the proposed mutant promoters as well as those promoters constructed in this research will yield much new information. These studies will precisely demonstrate whether the polyadenine regions interact directly with RNA polymerase and whether the presence of these regions alters the interactions of the enzyme with the promoter proper. These footprinting experiments and other experiments to measure initiation rates from these promoters may be carried out at different temperatures. This will allow us to more conclusively identify what step(s) in transcriptional initiation is most affected by the presence of the upstream curvature.
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VITA

Carl Feemster McAllister was born on October 30, 1954 in Tupelo, Mississippi. His family moved to Oxford, Mississippi in 1961, where he graduated from College Hill Academy in 1972. He attended the University of Mississippi and graduated Magna Cum Laude with a B.S. in Biological Science and a B.A. in Psychology in May of 1977. He later enrolled in the graduate program of the Biology Department at the University of Mississippi and was awarded the M.S. degree in Biological Science in August of 1982. His thesis was entitled "Characterization of Succinate Transport by Free-Living Forms of the Soybean Symbiont Rhizobium japonicum." This work received public forum at the 1983 national meeting of the American Society for Microbiology, and in the March, 1983 issue of the Journal of Bacteriology.

In August of 1983, he enrolled in the doctoral program in the Department of Microbiology at Louisiana State University. From 1983-1987 he was supported by an Alumni Federation Graduate Fellowship. In June of 1984 he married Sigrid Marie Klock and they have one child, Cameron Feemster McAllister, born May 8, 1987. Portions of his doctoral research have received public forum at the 1985-1988 national meetings of the American Society for Microbiology and at the 1987 Stony Brook Symposium - New Insights into the Regulation of Transcription. He was selected for the McCleskey Award at the 1986 annual meeting of the South Central Branch of the American Society for Microbiology.

A portion of his doctoral research is being presented in the Journal of Biological Chemistry (August 1988, in press). Carl is
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Major Field: Microbiology/Genetics

Title of Dissertation: The Role of Curved DNA in Promoter Selection by the Major Bacillus subtilis RNA Polymerase.

Approved:

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Major Professor and Chairman

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Dean of the Graduate School

EXAMINING COMMITTEE:

[Signature]

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Date of Examination:

Friday, July 15, 1988