The Role of Curved DNA in Bacillus Subtilis RNA Polymerase-Promoter Interactions.

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THE ROLE OF CURVED DNA IN
*BACILLUS SUBTILIS*
RNA POLYMERASE-PROMOTER INTERACTIONS

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
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Doctor of Philosophy

in

The Department of Biological Sciences

by

Murad M. Odeh
B.S., Al-Yarmouk University, 1990
M.S., Al-Yarmouk University, 1993
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DEDICATION

"To My Family, For Believing In Me,
For Being
A Constant Source Of Inspiration
And
For Showing Me The Way."
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ABSTRACT

Intrinsically curved DNA upstream of promoters was shown to affect the *Bacillus subtilis* RNA polymerase-promoter interactions leading to transcription initiation. The Alu156, a *B. subtilis* bacteriophage dependent on the curved DNA located immediately upstream of the -35 region, and modified forms of Alu156 were used. Promoters were examined using gel retardation and DNase I footprinting assays. DNase I footprinting analysis of the Alu156 promoter demonstrated a unique pattern of DNase I hypersensitive and protected sites characteristic of DNA wrapped around the protein. This wrapping was observed upstream of the curved DNA. To examine the nature of curved DNA, the native curved DNA in Alu156 was replaced with synthetic curved DNA containing one, two, or three adenine tracts (Alu1A, Alu2A, or Alu3A). While the levels of binding to RNA polymerase, DNA wrapping and transcription were greater in the Alu3A promoter than Alu1A and Alu2A promoters, the levels never equaled those observed with Alu156. Since the native curved DNA of Alu156 is highly rich in (A+T) and (A+T)-rich DNA immediately upstream of the promoter is known to function as an α binding site, promoters containing (A+T)-rich regions were examined. These promoters displayed a significant increase in promoter activity relative to promoters lacking the curved DNA but never equaled the original Alu156 promoter. These results suggested that both the (A+T)-content and curvature of the native curved DNA of Alu156 contribute to RNA polymerase binding and transcription. Using a combination of temperature and competitor DNA to control which transcription intermediates were formed, the RNA polymerase-promoter complex that was wrapped was identified as an unstable, closed promoter complex analogous to the RPC1 complex. These studies also identified a relatively stable, closed complex that lacked DNA wrapping and met the criteria for RPC2. Based on DNase I footprinting and transcription
assays, the presence of the curved DNA on Alu156 greatly stimulated the formation of the \( \text{RP}_{C1} \) and its subsequent isomerization to \( \text{RP}_{C2} \).
INTRODUCTION

Of the 4,000 genes in the typical bacterial genome, only a fraction are expressed at any given time. Bacteria are highly versatile and responsive organisms because they sense the levels of many metabolites and regulate their metabolic patterns accordingly by a wide variety of mechanisms. Regulating the concentration of a cellular protein involves a delicate balance of many processes. There are at least six potential points at which the amount of protein can be regulated: synthesis of the primary RNA transcript, post-transcription processing of mRNA, post-transcription modification, mRNA degradation, protein synthesis (translation), and protein degradation. Gene activity is regulated primarily at the level of transcription. In bacteria, many genes are clustered in units called operons. An operon consists of control sites (an operator and a promoter) and a set of structural genes. Initiation of transcription requires three major components: (1) DNA-dependent RNA polymerase; (2) a promoter, which is a DNA sequence at which RNA polymerase may bind, thereby leading to initiation of transcription; (3) regulator genes encoding proteins that interact with the operator and promoter sites to stimulate or inhibit transcription. Moreover, a number of other sequence-dependent, structural variations in the DNA (e.g., intrinsic DNA curvature and palindromes) may influence the binding of some proteins at DNA sites where important events in DNA metabolism (replication, recombination, transcription) are initiated or regulated.

DNA-dependent RNA polymerase. Transcription of the genetic information from DNA to RNA is carried out through the action of the enzyme RNA polymerase. RNA polymerase from Escherichia coli (E. coli) is a very large and complex enzyme consisting of at least four kinds of subunits. The subunit composition of the entire enzyme, called the holoenzyme, is $\alpha_2\beta\beta'\sigma$. The $\alpha$ subunit is 36,152 daltons, $\beta$ (150,619 daltons), $\beta'$ (155,162 daltons), and $\sigma$ (70,263 daltons for the major vegetative $\sigma$ subunit,
transcription initiation and then dissociates from the rest of the enzyme (Chamberlin, 1982; Helmann and Chamberlin, 1988). RNA polymerase without this subunit is called the core enzyme, $\alpha_2\beta\gamma\delta\epsilon$; it contains the catalytic site. The $\beta'$ subunit binds the DNA template, and the $\beta$ subunit binds ribonucleoside triphosphate substrates and the two subunits contribute to the formation of the active site (Mooney et al., 1998). Assembly of the enzyme itself is carried out by the smaller $\alpha$ subunits (Ishihama, 1981). Furthermore, these smaller subunits are involved in protein-protein interactions with positive regulators (Ishihama, 1992). Several studies have shown that C-terminal deletion mutations of rpoA, the gene for the $\alpha$ subunit, reduces transcription in vitro from several positively controlled promoters but did not affect core assembly. This suggested that core assembly requires the N-terminal domain of $\alpha$, while it is the C-terminal domain (CTD) which is involved in regulatory protein-RNA polymerase interactions (deHaseth et al., 1998). Additionally, E. coli RNA polymerase $\alpha$ subunit is involved in modulation of pausing, termination, and antitermination by transcription elongation factor NusA (Mooney et al., 1998).

Some E. coli promoters differ greatly from the standard promoters and the recognition of these promoters by RNA polymerase is mediated by different $\sigma$ factors (Doi and Wang, 1986; Losick et al., 1986). An example occurs in a set of genes called the heat shock genes, which are induced when the cell is under stress (e.g., a sudden increase in temperature). RNA polymerase binds to these promoters using the rpoH gene product, $\sigma^{32}$, and stimulates transcription of the heat shock genes (Grossman et al., 1984; Cowing et al., 1985; Fujita ad Fujita, 1987). E. coli synthesizes a group of heat shock proteins that enables it to adjust to this challenge (Neidhardt and Van Bogeler, 1987). Another heat shock $\sigma$ factor is involved in transcription at higher temperature. The rpoE gene product $\sigma^E$ ($\sigma^{24}$) regulates transcription of at least 10 genes. Rouviere et al. (1995) reported that $\sigma^E$ not only controls transcription from different genes, but also controls its own transcription from a $\sigma^E$-dependent promoter. A third $\sigma$ factor is called
σ^8 (σ^{18}) which is expressed in the stationary phase of bacterial growth (Tanaka et al., 1993). Arnosti and Chamberlin (1989) have shown that flagellar protein synthesis requires σ^F (σ^{28}). The σ^N (σ^{54}) is expressed under nitrogen starvation and is involved in the expression of certain genes so that several nitrogen sources can be utilized (Garcia et al., 1977). σ^N RNA polymerase is responsible in the expression of a number of diverse group of genes involved in metabolism.

**Sigma (σ) subunits of Bacillus subtilis RNA polymerase.** Hence, σ subunits are required only to ensure the specific recognition of the promoters by the RNA polymerase. The genes encoding these σ factors from *E. coli* and *Bacillus subtilis* (*B. subtilis*) have been sequenced and their primary structures have been identified (Helmann and Chamberlin, 1988). *B. subtilis* is a gram positive, sporulating bacterium. Sporulation is a time ordered process that occurs over a sufficient time period (7-10 hours) due to a cascade of modified RNA polymerases recognizing different sets of promoters (Losick and Pero, 1981; Doi, 1982). Several similarities in core subunit composition between RNA polymerases of *E. coli* and *B. subtilis* have been established. Studies of the major vegetative form of *B. subtilis* RNA polymerase, bearing the σ^A subunit, Eσ^A, have shown that this enzyme has an overlapping promoter specificity with the well characterized *E. coli* RNA polymerase (Achberger and Whiteley, 1981; Doi, 1982; Moran et al., 1982; Juang and Helmann, 1994). Additionally, initiation of transcription by the two enzymes is inhibited similarly by the drug rifampicin. On the other hand, there are some variances in subunit composition between these two RNA polymerases. Examples include the single 10 Kilodalton (kDa) *E. coli* rpoZ gene product omega (ω), while *B. subtilis* RNA polymerase contains two omega subunits. In addition, the *B. subtilis* RNA polymerase contains the 21 kDa delta (δ) protein encoded by the *B. subtilis* rpoE gene (Lampe et al., 1988). Another major difference between *E. coli* and *B. subtilis* RNA polymerases is that the latter has a larger number of σ factors which are involved essentially in regulating gene expression in a time-fashioned
order during sporulation. For example, $\sigma^A(\sigma^{43})$, the major subunit associated with RNA polymerase in vegetative cells controls the majority of cellular transcription (Losick et al., 1986).

Biochemical and genetic analysis have shown the presence of at least five additional $\sigma$ factors involved in endospore formation. The sequential progression for their appearances is as follows: $\sigma^H$, $\sigma^F$, $\sigma^E$, $\sigma^G$, and $\sigma^K$ subunits (Losick and Stragier, 1992). The $\sigma^A$ and $\sigma^H$ subunits are active before septum formation. However, $\sigma^F$, $\sigma^E$, $\sigma^G$, and $\sigma^K$, are cell type specific factors. $\sigma^F$ is produced in the mother cells and before the asymmetric cell division occurs. However, it remains inactive until after sporulation. The $\sigma^E$ subunit is active exclusively in the mother cell and it is needed for prespore formation. The $\sigma^G$ and $\sigma^K$ subunits are made in the forespore and the mother cell, respectively. The $\sigma^G$ subunit coordinates the transcription of forespore-specific gene, while $\sigma^K$ directs the transcription of mother cell-specific genes called the $cot$ genes which code for endospore $cot$ proteins. Alternate $\sigma$ factors are not only found in bacterial cells, but they are also found in vegetatively growing cells. $\sigma^D(\sigma^{28})$ (Wiggs et al., 1981) and $\sigma^B(\sigma^{37})$ (Haldenwang and Losick, 1980) were characterized. The role of $\sigma^D$ factor is to control a regulon of genes required for chemotaxis, morphogenesis, and other nutrient stress responses (Helmann and Chamberlin, 1987; Helmann et al., 1988). There is no distinct function for $\sigma^B$ (Binnie et al., 1986). However, Doi and Wang (1988) suggested that $\sigma^B$ might function during the stationary phase of growth and early sporulation. Recently, Haung and co-workers (1997) reported three additional minor sigma subunits, $\sigma^X$, $\sigma^Y$, and $\sigma^W$ based on the analysis of the genome of B. subtilis. Their role is to control gene expression during environmental stress.

SP01 and SP82, phages that infect B. subtilis, encode several new $\sigma$ factors that modify host RNA polymerase, leading to sequential expression of viral genes during infection. Expression of phage early genes is required for the transition to middle and late gene transcription. Transcription of the phage genes is temporally regulated since...
\(\sigma^A\) is required to express the gene for phage \(\sigma^{p28}\), and the \(\sigma^{p28}\) is required to express the genes for \(\sigma^{p33/34}\) (Duffy et al., 1975; Talkington and Pero, 1978; Lee and Pero, 1981). These \(\sigma\) subunits contribute to specific initiation by decreasing the affinity of RNA polymerase for the \(B.\ subtilis\) genome. The finding of multiple \(\sigma\) factors controlling specific promoter sites suggests a direct binding with the promoter sequence where RNA polymerase initiates transcription.

**Promoters.** Promoters are unique DNA sequences, commonly located upstream from the structural region of genes, and are involved in binding RNA polymerase for transcription initiation. Studies of sequence comparisons have shown that, in prokaryotes, two regions of nucleotide sequences are found in many, if not all, promoters and at precise sites relative to the point at which transcription begins. For the major form of RNA polymerase for many bacteria, such as \(E.\ coli\) and \(B.\ subtilis\), a sequence of TATAAT is found at locations 10 bases upstream from the transcription start site +1, the so called -10 region. The second region of conserved sequence is about 35 bases upstream from the start of transcription. The consensus sequence in the -35 region is TTGACA. The two consensus sequences are separated optimally by 17 base pairs (bp) of spacer DNA (Rosenberg and Court, 1979; Siebenlist et al., 1980; Hawly and McClure, 1983; Harley and Reynoldes, 1987). Different promoters interact with the RNA polymerase enzyme with different degrees of affinity, a so called "promoter strength." "Strong promoters" more frequently facilitate enzyme attachment than do "weak promoters". "Promoter strength" is directly related to the intrinsic DNA sequence of the promoter itself and to regulatory proteins, which may modulate interactions between certain promoters and RNA polymerase (Von Hippel et al., 1984). The -10 region of a promoter is involved directly in the rate of open complex formation. The -35 region is needed for promoter recognition and the initial binding by RNA polymerase (Gilbert, 1976). Furthermore, mutational studies performed by Kobayashi and co-workers (1990) have demonstrated that base substitutions in the -35 region affect
both the binding to RNA polymerase and open complex formation rate. Additionally, promoter specificity can be altered as a result of amino acid substitutions in the DNA binding domain of σ that recognizes promoter sequences (Schmidt et al., 1990). These findings were similar to results obtained by Doi and Wang (1986) in which holoenzyme containing such a mutant σ recognizes unique promoters with different consensus promoter sequences.

The consensus sequences of common promoters are recognized by the major vegetative RNA polymerases from both *E. coli* and *B. subtilis* (Moran et al., 1982; Galas et al., 1985). It has been reported that some promoters from *E. coli*, a gram-negative bacteria, can function in *B. subtilis*, a gram positive bacteria, using σ^A factor of RNA polymerase (Peschke et al., 1985). However, almost all *B. subtilis* promoters are highly utilized by *E. coli* σ^70 RNA polymerase both *in vivo* and *in vitro*. Thus, *E. coli* σ^70 RNA polymerase might be less sensitive than the *B. subtilis* σ^A to structural requirements in the DNA sequence adjacent to the -10 and -35 region.

Additionally, the promoters of some gram-positive species have some other conserved regions, such as a track of five adenine bases in the -3 and -7 regions (Graves and Rabinowitz, 1986). Recently, an (A+T)-rich region of 20 base pairs called the UP element, positioned upstream of the -35 region of the *E. coli* ribosomal RNA promoter *rrnBpI*, was shown to be implicated in transcription regulation (Ross et al., 1993). The UP elements are the binding sites for the α subunits. Deletion mutations of the carboxy-terminal domain (CTD) of the α subunits of RNA polymerase significantly reduces the binding activity of the enzyme, suggesting that an α subunit is involved in the regulation of transcription initiation at some promoters. Furthermore, *in vivo* and *in vitro* studies done by Fredrick et al. (1995) have shown that transcription from a *B. subtilis* flagellar promoter is stimulated by the UP element.

**Sequences associated with curved DNA.** A number of sequence-dependent structural variations in the DNA helix have been detected that may serve locally important
functions in DNA metabolism. For example, some sequences cause bending in the DNA helix. DNA curvature was first recognized in the mini-circle of mitochondrial DNA of *Leishmania tarantolae*, kinetoplast DNA (Marini et al., 1982). The anomalous electrophoretic mobility of the mini-circle has become closely identified with DNA curvature (Hagerman, 1984; Wu and Crothers, 1984; Trifonov, 1985). DNA bending has been confirmed by a variety of experiments such as polyacrylamide gel electrophoresis analysis, electron microscopy, X-ray crystallography, and chemical probes including DNase I digestion and hydroxy radical cleavage. Intrinsic bends are produced by runs of 4-9 adenine residues. Along the adenine tract (A-tract), there is a progressive narrowing in the minor groove from 5' to 3' leading to a deflection of the helix. A-tracts spaced in the helical repeat of the DNA are additive and contribute to overall curvature (Hagerman, 1984; Dieckmann, 1986; Koo et al., 1986). Most models have been refined to one which suggests that the DNA helix in the A-tract is characterized by base inclination in the form of negative roll (Haren et al., 1994). There is a progressive narrowing in the minor groove of the helix from the 5' end of the A-tract until it reaches a minimum at the 3' end of the A-tract. The minor groove widens in the segment of B-form DNA separating the A-tracts. Based on this model, researchers can accurately predict the direction and the degree of the curvature associated with the A-tract. However, according to X-ray crystallography, Goodsell et al. (1993) have developed a contradictory model which predicts the degree of both the curvature and the direction. A-tracts are not sequence-directed curvature but they are "straight" segments of DNA and they have zero roll. On the other hand, all other DNA is proposed to have a positive roll which results in a narrowing in the major groove across the B-form DNA separating A-tracts. In addition to intrinsic DNA curvature generated by A-tracts, there are some other unusual forms of DNA that have been observed to cause bending in DNA, for instance, when the sequence contains particular periodic dinucleotides (AG, CG, GA, or GC), bending in the DNA helix is observed (Bolshoy et al., 1991).
However, the degree of curvature developed by this type of "non-tract" bending is much smaller than for A-tracts (Fujimura, 1988, Bolshoy, et al., 1991). X-ray studies by Godsell and co-workers (1993) have shown that the GGCC sequence is another intrinsically curved element which is bent toward the major groove. A third element has also been reported to cause DNA bending. GGGCCC-containing DNA in the presence of divalent ions has shown a strong gel mobility anomaly indicative of DNA curvature (Brukner et al., 1994). The ionic effects are stronger for GGGCCC compared to A-tracts and the presence of ions alter the sequence-dependent dynamic feature of DNA.

Transcription initiation kinetics. Regulation of gene expression is a critical component for a bacterium to survive. This regulation occurs primarily at the level of transcription. Transcription initiation by RNA polymerase is a complex process which involves a series of defined biochemical intermediates (Chamberlin, 1974; VonHippel et al., 1984; McClure, 1985). A model was developed by Chamberlin (1974) using E. coli in his studies in which two kinetic intermediates were proposed.

\[ R + P \leftrightarrow R P_C \leftrightarrow R P_0 \leftrightarrow RNA\ synthesis \]

This model is called the bipartite model in which RNA polymerase (R) binds the promoter (P) and forms "closed complex" (RP_C) (Chamberlin, 1974). This is followed by isomerization of the latter into an "open complex" (RP_0). In the RP_0 complex, at least 12 base pairs are melted to form a transcription bubble, allowing the exposure of the template DNA strand to RNA polymerase for RNA synthesis (Siebenlist et al., 1980; Kirkegaard et al., 1983). At least two intermediates prior to formation of the open complex have been documented (Rosenberg et al., 1982; Roe et al., 1984; 1985; Buc and McClure, 1985; Duval-Valentin and Ehrlick, 1987). The two intermediates are included in the following modified rate equation:

\[ R + P \leftrightarrow R P_{C1} \leftrightarrow R P_{C2} \leftrightarrow R P_0 \leftrightarrow RNA\ synthesis \]
First, binding of RNA polymerase to the promoter to form the less stable first closed complex (RP\textsubscript{C1}) (Kadesch et al., 1982; Rosenberg et al., 1982), followed by the formation of a more stable second closed complex (RP\textsubscript{C2}) (Roe et al., 1984; Buc and McClure, 1985), in which incomplete unwinding of the DNA helix takes place. The RP\textsubscript{C2} complex is isomerized to the most stable open complex (RP\textsubscript{O}) leading to transcription initiation and RNA synthesis. Walter et al. (1967) used heparin, a polyanion with high affinity for RNA polymerase, to investigate the kinetics of transcription initiation. The two closed promoter complexes react differently with heparin. RP\textsubscript{C1} appears to be sensitive to challenge by heparin while RP\textsubscript{C2} is not. After the formation of RP\textsubscript{C2}, an open complex is formed. Since the rate of open promoter complexes formation is temperature dependent, decreasing the temperature inactivates the isomerization and retards their formation. On the other hand, increasing the temperature drives the isomerization from the initial closed complex to the open complex.

Abortive RNA synthesis has been recognized as an intermediate step between open complex formation and productive RNA synthesis (Carpouis and Gralla, 1980). E. coli RNA polymerase can experience multiple rounds of abortive initiation in which short pieces of RNA are released from the transcription complex. Repeatedly, when RNA chain reaches a length of 9 to 11 bases, the abortive initiation phase goes into the productive step, promoter clearance (Grachev and Zaychicov, 1980; Straney and Crothers, 1985a; Garpousis and Gralla, 1985). By promoter clearance, elongation phase of RNA formation starts after synthesis of the first phosphodiester bond. An important modification is the loss of a σ subunit, where the core enzyme without σ binds more strongly to the DNA template. RNA polymerase remains bound to its template until a termination signal is reached. Specific mechanisms exist to rescue the elongated RNA polymerase from arrested complexes and promoter progressive elongation of the RNA (Mooney et al., 1998).
Various techniques are applied to study specific RNA polymerase-promoter interactions. Filter binding assays, runoff transcription (Roa et al., 1984) and abortive initiation (Hawley and McClure, 1982) can be used. Recently, RNA polymerase-promoter interactions have been addressed by using other methods such as gel retardation analysis (Crothers, 1987; Garner and Revzin, 1986) and chemical probes including 1,10-phenanthroline-copper footprinting techniques (Spassky, 1992). Gel retardation analysis is utilized to detect the binding of RNA polymerase for promoters. The chemical probe, 1,10-phenanthroline-copper is a helpful tool in obtaining information on DNA form, solvent accessibility, and the presence of single-stranded DNA in open promoter complexes. There are additional chemical probes such as DNase I digestion and potassium permanganate used in exploring the nature of RNA polymerase-DNA interactions. Information concerning the location where the enzyme (protein) binds, DNA conformation as a result of protein binding, and the kinetic intermediates of transcription initiation have been obtained using DNase I footprinting analysis. \textit{In vivo} and \textit{in vitro} analysis of promoter DNA melting in open complexes (Sasse-Dwight and Gralla, 1989; Kainz and Roberts, 1992) have been investigated using potassium permanganate, which is recognized to react selectively with pyrimidines in single-stranded DNA.

\textbf{Proteins involved in transcription regulation.} Prokaryotes adjust the amount of proteins synthesized in a variety of ways. The expression of most genes is regulated essentially at the level of transcription. Regulator genes encode proteins that interact with the operator and promoter sites to stimulate or inhibit transcription. The lactose operon of \textit{E. coli} displays positive regulation by the catabolite gene activator protein (CAP) (Zinkel and Crothers, 1990). When cyclic AMP (cAMP) concentrations are high (glucose concentrations are low), cAMP binds CAP forming a complex in that binds to a specific site in the promoter and stimulates transcription of the \textit{lac} operon by enhancing the binding of RNA polymerase to the promoter. This leads to the synthesis of lactose-
metabolizing enzymes. On the other hand, cAMP concentrations decrease in the presence of glucose, thus the cAMP-CAP complex can not form, thereby limiting the expression of the lac genes and suppressing secondary sugar utilization. DNA bending is involved in the stimulation of transcription by CAP. The bend induced by CAP is estimated to be about 100° to 130° (Thomson and Landy, 1988; Zinkle and Crothers, 1990).

Another example of a regulatory protein is the LacI gene product encoded in the lac operon (Jacob and Monod, 1961). The regulator gene of the lactose operon encodes the lac repressor which has two binding sites. One site binds to the operator overlapping the lac promoter thereby preventing initiation of transcription. The second site is the target for the inducer (allolactose) or isopropylthiogalactoside (IPTG). If an inducer is present, it binds to the repressor forming an inducer-repressor complex while inducing conformational changes in the structure of the repressor. Therefore, the repressor can not bind to the operator allowing RNA polymerase to bind to the promoter and initiate transcription.

*B. subtilis* phage φ29 encodes a protein called p4 which also bends DNA. Switching from early to late transcription requires the synthesis of the p4 protein at early stages of infection. Information about the direct interaction between protein p4 and RNA polymerase at the PA3 promoter has been collected (Neuz *et al*., 1991; 1992; Serrano *et al*., 1991). The presence of p4 activates transcription of late genes from a single promoter PA3 by stabilizing the binding of *B. subtilis* RNA polymerase to the promoter as a closed complex (Neuz *et al*., 1992).

The N4ssB, a single-stranded DNA binding protein of *E. coli* bacteriophage N4 is another example of a transcription factor that interacts with RNA polymerase. Miller and his colleges (1997) have shown that N4ssB interacts with the carboxy-terminus of the RNA polymerase β' subunit and activates transcription by the *E. coli* σ70 RNA polymerase at the N4 late promoter.
Another element that might influence transcription initiation is DNA structural organization. Experimental studies have demonstrated that DNA supercoiling can either activate or repress transcription from some promoters, while having no significance on many other promoters (Pruss and Darlica, 1989). The binding of RNA polymerase to promoter sequences and the stabilization of activators to DNA might be induced by DNA supercoiling. Gralla (1989) has reported that supercoiled DNA assists transcription initiation at the lacP1 promoter of E. coli, similar to a CAP-dependent fashion.

The effects of some regulatory DNA sequences can be exerted from a distance. Distant DNA sequences can be brought into proximity by DNA looping, mediated by specific protein-protein and protein-DNA interactions. This property made the gal operon an ideal unit for the study of gene expression. There are two-overlapped promoters, P1 and P2, and two operators, an internal (O1) and an external one (O2). Oe and O1 are located upstream and downstream of the two-overlapped promoters, respectively (Irani et al., 1983; Adhya, 1987; Mandal et al., 1990). When galactose levels are low and glucose levels are high, the gal repressor binds to both O1 and Oe and brings these sites together by forming a DNA loop. Consequently, RNA polymerase is prevented from binding to the structured promoters, and transcription initiation is inhibited. Repression of transcription by DNA looping is also found in the lac operon where the two functional operators in the lac operon are positioned downstream of a single promoter (Mossing and Record, 1986; Kramer et al., 1987).

Finally, intrinsically curved DNA sequences have been detected in various promoters (Verde et al., 1981; Bauer et al., 1988; McAllister and Achberger, 1988; Thomson and Landy, 1988; Zinkle and Crothers, 1990). The curved DNA is often observed as runs of adenine residues in phase with the helical repeat of 10.5 bp per turn of the B-form DNA. It has been documented that DNA bending, wrapping, and looping are affected by sequence-dependent DNA curvatures (Lomond and Traverse, 1983;
Bossi and Smith, 1984; Galas et al., 1985; Plaskon and Wartell, 1987; McAllister and Achberger, 1988; Hsu et al., 1991; Tanaka et al., 1991).

The effect of δ subunit on transcription initiation. *B. subtilis* RNA polymerase contains a catalytic core (α₂ββ'), one of the thirteen σ factors, the two omega subunits, and the 21 kDa delta (δ) subunit. The σ subunit is composed of two major regions: an amino-terminal domain (95 amino acids) with a uniform charge distribution that binds to RNA polymerase and the negatively charged, 69-amino acid carboxy terminal domain (Lopez deSaro et al., 1995). Since vegetative *B. subtilis* RNA polymerase is associated with a 43 kDa σ factor (σ^A^-) and an additional subunit δ, it has been speculated that the functions exerted by the *E. coli* σ^70 can be accomplished by both *B. subtilis* components, σ^A^- and δ. Both *B. subtilis* σ^A^- and *E. coli* σ^70 appear to recognize similar promoter sequences and to be functionally homologous. Amino acid sequence comparison between both subunits has revealed an extensive amino acid identity allowing a 245-amino acid gap introduced between conserved region I and region II of the σ^A^- subunit (i.e., an expanse of 245 amino acids separates region I from region II in the *E. coli* σ^70 subunit, while the two regions are almost contiguous in the *B. subtilis* σ^A^- subunit) (Gitt et al., 1985; Lopez deSaro et al., 1995). This stretch of 245 amino acids might be involved in decreasing the binding of RNA polymerase at non-promoter and weak promoter containing DNA sequences since this function has been related to the *E. coli* σ^70 subunit but not to the *B. subtilis* σ^A^- subunit. Non-specific contacts of RNA polymerase to weak and non-promoter DNA fragments are reduced by the *B. subtilis* δ factor (Achberger et al., 1982), the δ subunit of *B. subtilis* might be functionally homologous to the 245-amino acid stretch in the *E. coli* σ^70 subunit. In the RNA polymerase of *B. subtilis*, two subunits participate in providing optimal distinction between promoter and non-promoter sites. Pero et al. (1975) identified δ factor as a host encoded protein needed for *in vitro* transcription by the RNA polymerase from phage SP01-infected cells. The specific *in vitro* transcription of phage SP82 DNA by
phage-modified and host cell polymerases required the host δ protein (Spiegelman et al., 1978; Achberger and Whitely, 1981; Achberger et al., 1982). δ factor appears to limit the interactions between polymerase and DNA, possibly by altering the conformation of the polymerase, thus reducing the number of sites on the DNA that function as promoters (Achberger et al., 1982) and destabilizing complexes formed at weak promoters (Dobinson and Spiegelman, 1987). These data indicated the necessity of σ and δ subunits for promoter selectivity and transcription initiation. DNase I footprinting and methylation protection analysis at an early gene promoter of phage SP82 have shown that δ factor acts prior to transcription initiation to enhance promoter selectivity by changing RNA polymerase-promoter contact in non-initiated complexes but not in initiated ones (Achberger et al., 1982). Recently, data from DNase I footprinting studies and potassium permanganate probing have demonstrated that δ subunit significantly decreased RNA polymerase-promoter interactions and open complex formation at both the ilv-leu (P_{ilv}) and trns (P_{trns}) promoters. Core enzyme-σ-δ (Eσδ) favors the formation of closed complexes and inhibits promoter melting, whereas Eσ forms open complexes at both promoters. Despite the inhibitory effect of δ subunit on open complex formation, it enhances core enzyme recycling (Juang and Helmann, 1994; 1995).

Curved DNA in prokaryote promoters. Several DNA elements are responsible for promoter strength or the relative effectiveness of transcription initiation. Most important are the -10 and -35 conserved sequences and the spacer sequence. Likewise, intrinsically curved DNA sequence generated by a run of A-tract positioned upstream of the -35 region is a major determinant (Lomond and Traverse, 1983; Bossi and Smith, 1984; Galas et al., 1985; Plaskon and Wartell, 1987; McAllister and Achberger, 1988; Hsu et al., 1991; Tanaka et al., 1991). A correlation between curvature and transcription has been established in several E. coli ribosomal and tRNA promoters (Bauer et al., 1988), the ompF promoter (Verde et al., 1981), the his and lpp promoters (Verde et al., 1981),
the bla promoter from pUC19 (Ohyama et al., 1992), and the Alu156 promoter from B. subtilis phage SP82 (McAllister and Achberger, 1988).

In vivo studies performed by Bracco and co-workers (1989) have shown that transcription activity of E. coli gal promoter can be restored if the CAP site is replaced by synthetic curved DNA (i.e., some synthetic curved DNA sequences can act as transcription activators in E. coli, at least under particular in vivo conditions). These findings were consistent with the results obtained from experiments using synthetic curved DNA in the lac promoter (Gartenberg and Crothers, 1991). Moreover, Ellinger et al. (1994) have demonstrated the correlation between curved DNA and promoter activity in E. coli. In his work, synthetic promoters containing curved DNA, which are known to be rate limited at different steps during transcription, were used. Promoter activity was increased in vivo in promoters which are rate limited at early steps in transcription initiation (e.g., closed complex formation). This enhancement of promoter activity is due to the A-tracts facilitating interactions between limiting RNA polymerase and the promoters thus increasing the rate of open complex formation. By contrast, promoters which are limited in late steps of transcription initiation (e.g., promoter clearance or elongation) were inhibited by the curved DNA as a result of slowing the escape of the polymerase elongation complexes from the promoter. In the same study, phasing of the A-tracts relative to the -35 region was important for promoter function. A periodic change of promoter activity was noticed with the displacement of the A-tracts from the core promoter sequence. The position that caused maximal activation at one promoter caused maximum inhibition at another indicating the importance of the phasing of these sequence elements. These observations suggest that the same molecular interactions give rise to both reduction and enhancement of promoter function. These observations are in agreement with previous studies using B. subtilis RNA polymerase (McAllister and Achberger, 1989) in which proper spatial arrangement of A-tracts
relative to core promoter are of higher significance than the linear distance between these elements and the core promoter.

The presence of (A+T)-rich DNA upstream of the -35 region, resulting in DNA bending, has been shown for a variety of σ-containing holoenzymes. These (A+T)-rich areas have been shown to require for effective transcription of B. subtilis promoters. An E. coli promoter containing an (A+T)-rich region upstream of the core promoter region was found to be very efficient in vivo in B. subtilis (Peschke et al., 1985). A significant drop in transcription effectiveness in B. subtilis was observed when deletions in the (A+T)-rich regions upstream of the -35 region in Streptococcus pneumoniae mal promoter were introduced (Espinosa et al., 1984). This observation was in agreement with previous studies in which deletion in the (A+T)-rich region of B. subtilis SpoVG promoter dramatically decreased promoter activity in vivo due to the loss of DNA curvature (Banner et al., 1983). Moreover, serious reductions in in vitro transcription were detected from several B. subtilis promoters lacking upstream (A+T)-rich regions (e.g., promoters for spoVG, veg, etc.).

Previous work in the Lab. The significance of sequence-dependent DNA curvature in transcription activation has been addressed in our laboratory. Promoters containing upstream sequence-dependent DNA curvature have been well-documented. Curved DNA facilitates protein mediated DNA bending and structuring. Alul56, a promoter from B. subtilis bacteriophage SP82 that is dependent on curved DNA for efficient function was studied. McAllister and Achberger (1988) have reported that deletion of the curved DNA resulted in greater than a 10-fold decrease in transcription in vivo. A remarkable finding was that DNA curvature must be oriented on the correct face of the DNA helix to maximize stimulation of promoter activity. Double oligonucleotide insertions between the -35 region and the curved DNA (i.e. starting at -40) were used to displace the curve relative to the rest of promoter. For each base pair inserted, the curved DNA was rotated by about 34° relative to the core promoter. In vitro studies
were performed to investigate the effect of DNA curvature on transcription initiation by *B. subtilis* RNA polymerase as a function of temperature. The highest levels of transcription were observed for the original Alul56. Altered promoters that showed the greatest number of transcripts were consistently the +11 and +21 insertion promoters in which the curved DNA was on the same side of the helix as Alul56. However, promoters with insertion of +6, +15, +15, +17, and +29 where the curve was on the opposite side of the helix were among the weakest promoters and formed the fewest open promoter complexes (Stemke, 1993). These results were in agreement with earlier studies (McAllister and Achberger, 1989) in which promoters with properly aligned curved DNA (*e.g.*, Alul56 and the +11 and +21 insertion promoters) bound RNA polymerase tightly and were efficiently transcribed *in vitro* and *in vivo*. Promoters in which the orientation of the curved DNA was misaligned were not efficiently transcribed.

Since gene expression from some *B. subtilis* promoters is highly dependent upon the curved regions located upstream of the promoter consensus sequence, characterization of the mechanism for transcription stimulation was of interest. The original Alul56 and a collection of Alul56-derived promoters recognized by σ^A^-RNA polymerase were used to examine the effect of curved DNA on RNA polymerase binding and the conformational changes in the DNA leading to transcription. DNase I digestion patterns demonstrated that RNA polymerase wraps DNA upstream of the promoter around the enzyme (McAllister and Achberger, 1989, Cheng; 1996). While wrapping was more evident in promoters containing curved DNA sequences, interaction of RNA polymerase with DNA upstream of the promoter was observed in all promoters. Alul56 promoter displayed repeated DNase I hypersensitive sites separated by regions weakly protected from DNase I cleavage. The approximately 10 base pair periodicity of the repeated enhancement and protection sites is indicative of DNA wrapping around the RNA polymerase. Double-stranded oligonucleotide insertions between the -35 region and the first run of the A-tracts did not eliminate DNA wrapping. Wrapping was more

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prominent in promoters in which the curved DNA was properly aligned with promoter. Titration of the reactions with NaCl disrupted DNA wrapping while the RNA polymerase remained bound to the promoter suggesting that electrostatic interactions hold the upstream DNA to the enzyme. While no detectable open promoter complexes are formed at 0°C, DNase I digestion patterns consistent with DNA wrapping was observed. It was concluded that DNA structuring, including the wrapping of DNA around RNA polymerase, occurs prior to open complex formation and may contribute to strand separation and transcription initiation (Cheng, 1996).

Previous experiments in our lab have used the original lambda (λ) phage promoters, \( P_R \) and \( P_L \), and hybrid promoters containing the curved DNA from the Alu156 and Bal129 promoters to test the effect of curved DNA on the binding of \( E. coli \) RNA polymerase to these promoters. Results showed that hybrid promoters with curved DNA were bound by RNA polymerase more efficiently than the original \( \lambda \) promoters when the DNA templates were supercoiled. DNase I footprinting of \( E. coli \) \( \sigma^{70} \)-RNA polymerase bound to \( P_R \) promoter containing the added curved DNA provided evidence for DNA structuring by \( E. coli \) RNA polymerase. DNase I digestion pattern was indicative of wrapping of the curved DNA upstream of the promoter around the RNA polymerase with the DNA bent in the same direction as the intrinsic curve (Nickerson and Achberger, 1995).

Other studies examined the binding of \( E. coli \) \( \sigma^{70} \)-RNA polymerase to the original Alu156 and altered promoters in which the curved DNA immediately upstream of the -35 region was deleted or exchanged for non-curved DNA rich in (A+T). DNase I cleavage patterns observed for the Alu156 promoter bound to RNA polymerase were consistent with wrapping of DNA upstream of the -35 region around RNA polymerase. Upon addition of heparin, a decrease in bound complexes was observed and the DNase I cleavage pattern indicative of DNA wrapping was completely lost after 30 seconds (Jazbi, 1997). This is consistent with the loss of wrapped DNA-RNA polymerase.
complexes. Upstream interactions between DNA upstream of the promoter and RNA polymerase were lost above 150 mM NaCl, indicating the electrostatic, sequence-independent nature of these interactions. Results of gel retardation assays have shown that promoters containing curved DNA displayed the highest binding to RNA polymerase relative to the promoters containing an (A+T)-rich region or an UP element of ribosomal RNA promoter \textit{rrnBP1}.

Efficiency of transcription for the original promoter, Alu156, and the altered ones was investigated using single-round, runoff transcription assay. A greater number of transcripts were formed by the original Alu156 at low temperatures relative to promoters lacking the curved DNA. These data suggest that the curved DNA facilitates DNA wrapping around RNA polymerase and stimulates the formation of heparin resistant complexes that isomerize quickly into open complexes.

The primary interest of this research was to investigate the role of sequence-dependent DNA curvature on the interaction between \textit{B. subtilis} RNA polymerase and DNA sequences upstream of the promoter. In the work presented here, a promoter dependent on curved DNA for efficient function was selected. This promoter, Alu156, was isolated from the \textit{B. subtilis} bacteriophage SP82. Derivatives of the Alu156 promoter in which the curved DNA immediately upstream of the -35 region was altered or exchanged for non-curved DNA rich in (A+T) were available. A second set of promoters were constructed in which the native curved DNA was replaced with synthetic DNA containing one, two, or three A-tr tricks (Alu1A, Alu2A, Alu3A), positioned in phase with the helical repeat of the DNA (i.e., these synthetic runs of adenine tracts were positioned at the same side of the DNA helix relative to the core promoter). Additionally, two other derivatives of the synthetic promoter Alu3A were constructed in which the orientation of the curved DNA immediately upstream of the -35 region was altered. Furthermore, the identification of transcription initiation complexes was addressed. Finally, the effect of enzyme subunit composition, specifically the $\delta$
subunit, on RNA polymerase-promoter interaction and transcription initiation was investigated. The results provide direct evidence that *B. subtilis* RNA polymerase wraps DNA found upstream of the promoter sequence around itself. The wrapped RNA polymerase-promoter complex was most evident with promoters possessing curved DNA. Based on the nature of RNA polymerase interactions, a model has been proposed.
MATERIALS AND METHODS

Chemicals. Restriction endonucleases were purchased from either Bethesda Research Laboratories or New England Biolabs. PCR reagents were purchased by Perkin Elmer. DNase I was purchased from Boehringer Mannheim GmbH. Potassium permanganate was obtained from Sigma Chemical Company. The [γ32P] and [α32P] ATP were provided by New England Nuclear, Dupont. All other chemicals were of the highest quality available.

Bacterial transformation and plasmid isolation. Escherichia coli strain DH5αMCR was used as a transformation host. E. coli cells were made competent by the CaCl₂ method of Lederberg and Cohen (1974). About 50 μl of competent cells were mixed with 20-50 ng plasmid in a sterile test tube, cooled on ice for 20 min and then heat shocked at 42°C for 2 min. The heat shocked cells were briefly placed on ice, diluted with L-broth, and incubated at 37°C with shaking for 90 min. E. coli cells were spread onto solid medium containing 50 μg/ml ampicillin. The plasmid content of ampicillin resistant colonies was examined using the alkaline-lysis method of Birnboim and Doly (1979). Large scale plasmid DNA isolation was performed, followed by cesium chloride equilibrium density gradient separation in the presence of ethidium bromide in order to isolate highly purified plasmid DNA. The purified DNA was suspended in sterile distilled water and quantified by spectrophotometry at 260 nm.

Construction of Alu156-derived promoters. The original Alu156 promoter was from Bacillus subtilis phage, SP82 (McAllister and Achberger, 1988). A series of altered Alu156 promoters were constructed. In one set of promoters, the native curved DNA was exchanged for synthetic DNA containing one, two, or three adenine tracts (A-tracts) phased with the helical repeat of B-form DNA. The Alu2A promoter with two A-tracts was constructed by using the Alu156 promoter as a template and PCR was used to amplify the promoter region. The Alu2A forward primer (5'-GCGAATTCAAAAAAGG
ATCCAAAAATGCCTTGACCTCTCTCCTACGAGGTGTG-3') was a 50-mer containing two adenine tracts with a BamHI site located between the two A-tracts and an EcoRI site at the 5’-end of the primer. The M13/pUC Sequencing Primer (-47) 24-mer (New England Biolabs) was used as the reverse primer. The resulting product lacked the native curved DNA but contained two synthetic A-tracts forming the Alu2A promoter. This constructed promoter was cloned into the pUC8 plasmid. Finally, a 101 base pair DNA fragment excised from the AluExt promoter, (McAllister and Achberger, 1988) was inserted at the EcoRI site.

The Alu1A promoter containing a single A-tract was constructed by removing one A-tract from the Alu2A promoter using BamHI. The new promoter was cloned into the pUC8 plasmid and the 101 base pair EcoRI fragment from AluExt was inserted at the EcoRI site.

To construct the promoter with three A-tracts, Alu2A was used as a template. The Alu3A forward primer (5’-GCGAATTCAAAAATGGGCCAAAAAGGATCCAAAATGCG-3’) was designed to add one A-tract and an EcoRI site to the 5’ end of the Alu2A. PCR was used to amplify the promoter using the M13/pUC Sequencing Primer (-47) 24-mer as the reverse primer. The resulting promoter referred as to Alu3A was cloned into the pUC8 plasmid, and the 101 base pair EcoRI fragment was inserted.

PCR amplification for all promoters was performed in a Perkin Elmer Model 480 thermal cycler using for 25 cycle at 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min. Ampli-Taq DNA polymerase was used in the PCR reactions. Alu2A and Alu3A primers were synthesized in the Gene Lab, Louisiana State University. All promoter constructs, Alu1A, Alu2A, and Alu3A were sequenced using the Circumvent Thermal Cycle Sequencing Kit (New England Biolabs) to ensure that there were no mutations in the promoter sequence other than those intended.

To disrupt DNA curvature with minimal changes in DNA sequence of Alu156, the AluUnc promoter was constructed (Jazbi, 1997). In the AluUnc promoter, an
adenine nucleotide was replaced by a thymine in each run of A-tract and a thymine was replaced by an adenine in the thymine run using site directed mutagenesis (Chen and Przybyla, 1994). This technique required two rounds of PCR amplification. In the first cycle, desired mutations were introduced in a pUC8 derivative containing the original Alu156 promoter by using the AluUnc primer (5'-GCTAATATTCTGAATATAATTGCAATAAGTTGGAC-3') and the M13/pUC Sequencing Primer (-47) 24-mer (New England Biolabs). The first PCR product was gel eluted, ethanol precipitated, and desalted on Sephadex G50 (Pharmacea) spin column (Neal and Florini, 1973) to remove traces of salts and excess primers. Purified DNA fragments were used directly as a primer along with M13/pUC Reverse Sequencing Primer (-48) 24-mer (New England Biolabs) to undergo a second cycle of amplification using the Alu156 promoter as a template. The second amplified DNA fragments were double digested with EcoRI and HindIII, gel purified and ligated to pUC8 that had been digested with the same enzymes. Another promoter, AluUp, was constructed to investigate the role of the (A+T)-rich region upstream of the promoter (Jazbi, 1997). In this promoter, the curved DNA region was replaced by an (A+T)-rich sequence using PCR. The UP element (Ross et al., 1993), which is an (A+T)-rich region immediately upstream of E. coli rrnBp1 promoter, was inserted upstream of the -35 region of the wild type Alu156 promoter. The AluUp promoter was constructed using the original Alu156 as a template, the AluUp primer as one primer (5'-GCGAATCAGAAAATTATTTTAAATTGTGTGGATTTCTCTACGAGGTGT-3'), and the M13/pUC Sequencing Primer (-47) 24-mer (New England Biolabs) as the other primer. Purified PCR products were ligated to pUC8 after each was digested with EcoRI and HindIII. The 101 base pair EcoRI fragment from AluExt was inserted upstream of the promoter. AluUnc and AluUp primers were synthesized in the Gene Lab, Louisiana State University, and the presence of only the desired changes in the constructed promoters was confirmed by using the Circumvent Thermal Cycle Sequencing Kit (New England Biolabs).
Construction of Alu3A-derived promoters. Because proper rotational orientation of DNA curvature is critical for stimulation of RNA polymerase binding by the upstream curved DNA, two derivatives of Alu3A promoter, Alu3A(+1) and Alu3A(-1), were constructed in which the curved DNA immediately upstream of the -35 region was moved by one base pair (bp). For the first promoter, Alu3A(+1), a 1 bp insertion was created using site directed mutagenesis (Chen and Przybyla, 1994). Two rounds of PCR were required in this method. In the first round, the M13/pUC Sequencing Primer (-47) 24-mer (New England Biolabs) and the Alu3A(+1) primer (5'-TCCAAAAATGTCGTTGACTTTCTC-3') were used to amplify the DNA fragments containing the mutation. Plasmid pUC8, containing the Alu3A promoter, was used as a template. The Alu3A(+1) primer was 24 bases long and identical in sequence to the -35 region of Alu3A promoter with the exception that it contained 1 additional thymine between the -35 region and the curved DNA. These PCR products were gel purified, ethanol precipitated and desalted for the second round of PCR. The purified DNA fragments and M13/pUC Reverse Sequencing Primer (-48) 24-mer (New England Biolabs) were used as primers for the second round of amplification. The second PCR products were double digested with EcoRI and HindIII. The restriction fragments were gel purified, ethanol precipitated, desalted and ligated to pUC8 digested with the same enzymes as the PCR product.

For the second mutation promoter, Alu3A(-1), which is identical in sequence to the -35 region of Alu3A promoter but missing a cytosine between the -35 region and the first run of the adenine tract. This deletion was made following the same procedures used for Alu3A(+1) except that the Alu3A(-1) primer (5'-TCCAAAAATGTTGACCTTTCTC-3') was used instead of Alu3A(+1) primer. The Alu3A(+1) and Alu3A(-1) promoters were sequenced using the Circumvent Thermal Cycle Sequencing Kit (New England Biolabs) to confirm no other changes were made in the promoters. The 101 base pair DNA fragment from AluExt was inserted in both promoters after their digestion with EcoRI.
Electrophoretic analysis of curved DNA containing promoters. DNA curvature was analyzed and confirmed for the various promoter fragments by comparing the actual size of DNA fragments to their apparent size based on electrophoretic mobility of the DNA fragments in 10% polyacrylamide gels (30:0.5 acrylamide:bis-acrylamide) at room temperature (25°C). In this gel system, a Tris-boric acid-EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, and 2.5 mM disodium ethylenediaminetetraacetic acid (EDTA) pH 7.9) buffer solution was used.

Primer labeling. Primers were 5'-phosphorylated with T4 polynucleotide kinase (New England Biolabs). For each reaction, 30 mCi [γ-32P] ATP (Dupont, New England Nuclear), 20 pmol of primer, 10 units of kinase and an enzyme buffer provided by the manufacturer were mixed and incubated at 37°C for 30 min. Before storing the reaction at -20°C, reactions were heated to 90°C for 5 min to inactivate the kinase.

DNA amplification by PCR. Derivatives of pUC8 containing each of the promoters were used as templates along with M13/pUC Reverse Sequencing Primer (-48) 24-mer and the M13/pUC Sequencing Primer (-47) 24-mer (New England Biolabs). Only one of the two primers was 5'-end labeled with [γ-32P] ATP (as described above). In each PCR reaction, 2.5 μl of supplied 10X PCR buffer, 2 μl 25 mM MgCl2, 2 μl of a mixture containing 2.5 mM of each deoxynucleotide substrates, 0.1 μl Ampli-Taq DNA polymerase, 4 μl 5'-end-end labeled primer (0.8 μM), 1 μl second primer (20 μM), 2 μl DNA template (0.2 ng/μl) and 11.5 μl water were mixed. PCR amplification reactions were run in a Perkin Elmer Model 480 thermal cycler for 25 cycles under the following conditions: 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min. After amplification, PCR samples were purified in a spin column using Sephadex G-50 (Pharmacea Biotech) (Neal and Florini, 1973).

Bacillus subtilis RNA polymerase isolation. Core σ RNA polymerase was isolated from B. subtilis 168 (Achberger and Whiteley, 1981). B. subtilis 168 was grown aerobically in 1/2 L-broth (5 g/l Bacto tryptone, 5 g/l NaCl, 2.5 g/l Bacto yeast extract)
to an optical density of between 1.2-1.4 at 600 nm. Approximately 50 g of cells (wet weight) were used for isolation. For each gram of cells, the cells were suspended in 0.2 ml 10 mM Tris-HCl (pH 7.9), 2.5 ml buffer A (10 mM MgCl₂, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, PMSF), 1 mg lysozyme, and an additional 1 mg PMSF for each gram of cells. Sonication was used to lyse the cells and lysates were centrifuged in a Beckman J2-21 centrifuge with the JA-20 rotor at 15,000 rpm for 15 min. To the supernatant fraction, 16.2 ml of 30% polyethylene glycol 8000, PEG (BDH chemicals Ltd) and 4.8 ml of 20% dextran T500 (Pharmacia) were added simultaneously per 42 ml of crude supernatant for PEG-dextran phase partitioning of enzyme. The solution was centrifuged and the RNA polymerase was eluted from the resulting dextran pellet with the same solution containing 9.12 g NaCl per 42 ml of original crude extract. Excess PEG was removed by the addition of 16.5 g ammonium sulfate per 100 ml extract. RNA polymerase was then precipitated using 150 ml of 35% ammonium sulfate and 23.5 g of crystalline ammonium sulfate and collected by centrifugation. The ammonium sulfate precipitate containing RNA polymerase was solubilized in a minimal volume of 10 mM Tris-HCl buffer (pH 7.9), 15% glycerol, 1 mM EDTA, 1 mM PMSF, 5 mM 2-mercaptoethanol, and 0.5 M NaCl. This aqueous solution was loaded onto a chromatography column (Bio Gel A-1.5m, Bio Rad laboratories) at 4°C. Fractions were analyzed initially by absorbance at 280 nm and then samples with high absorbances were assayed using an in vitro transcription assay (Spiegelman et al., 1978). Fractions possessing enzymatic activity were concentrated in an Amicon (model 202) filtration system under 42 psi nitrogen gas. After the sample was loaded on the column, the column was eluted with a continuous NaCl gradient from 0.5 to 0.6 M NaCl. To determine subunit composition and purity of the isolated polymerase, fractions containing RNA polymerase activity were analyzed by sodium dodecyl sulfide-polyacrylamide gel electrophoresis (SDS-PAGE). Active fractions with core subunits and σA were pooled, concentrated with 35% glycerol, and stored at -20°C.
The δ subunit of *B. subtilis* was isolated from the core δ RNA polymerase fractions collected during DNA cellulose chromatography. DNA cellulose column fractions with high concentration of δ were denatured in 6 M urea, 25 mM NaCl, 10 mM Tris-HCl (pH 7.9), and 2 mM EDTA. The subunits were passed through a phosphocellulose column (Whatman) which had previously been equilibrated with the same buffer (Achberger and Whiteley, 1981). All polymerase subunits, other than the δ subunit and α subunits were retained on the column. The column fractions containing δ and α subunits was dialyzed against 15% glycerol, 100 mM NaCl, 10 mM Tris-HCl (pH 7.9), and 1 mM EDTA in order to remove urea. The α and δ subunits were separated by DEAE Sephadex A 25 column chromatography (Pharmacia, Inc.).

Concentration of RNA polymerase was determined using the Bio-Rad protein assay. *B. subtilis* RNA polymerase concentration was compared with gamma-globulin as a reference protein. The polymerase activity was measured by [3H] UTP incorporation into transcription products (Spiegelman et al., 1978). Reaction mixtures consisted of 40 mM Tris-HCl (pH 7.9), 10 mM MgCl2, 50 mM NaCl, 20 μg DNA (typically phage SP82 DNA), 1.5 to 96 μg of RNA polymerase and 0.8 mM each of GTP, ATP, CTP, 8 μM UTP, and 1.25 μCi of [3H] UTP in a total volume of 500 μl. One ml of 10% trichloroacetic acid (TCA) and 100 μl (2 mg/ml) of *Saccharomyces cerevisiae* RNA were added to the reactions after incubation at 37°C for 10 min. Before washing with 5% TCA, reaction mixtures were filtered through glass filters (Schleicher and Schenll). Dried filters were placed in scintillation fluid and counted in a scintillation counter (Beckman LS6800).

A transcription titration curve was established using *E. coli* phage T7 DNA as a template to determine the amount of δ subunit needed to saturate core-α and form the holoenzyme. Since δ is involved in promoter selectivity, it significantly inhibits *B. subtilis* RNA polymerase transcription activity on *E. coli* phage T7 promoters. Increasing the concentration of δ with a defined concentration of core-α RNA
polymerase was used to perform the transcription assay. The concentration of \( \delta \) that maximally inhibits transcription defines the saturating concentration (Achberger and Whiteley, 1981).

**Gel-mobility shift analysis.** To determine the relative affinity of *B. subtilis* RNA polymerase for different promoters, gel retardation analysis was chosen as described by Ausubel et al. (1989) with a few modifications. Reactions containing 5'-end labeled DNA, *B. subtilis* RNA polymerase, 1 \( \mu \)g noncompetitive DNA (pUC18), were incubated in a binding buffer [40 mM Tris-HCl (pH 7.9), 10 mM MgCl\(_2\), and 50 mM NaCl] for 10 min at 37°C. Loading dye (26% ficoll and 0.1% bromophenol blue) was added prior to electrophoresis on 4% polyacrylamide gel (acrylamide to bis-acrylamide, 30:2). Gel retardation experiments were performed in high ionic strength running buffer (50 mM NaCl, 400 mM glycine, and 2 mM EDTA, pH 8.5), and gels were run at 150 volts at 25°C for 2 hours. Gels were then transferred to Whatman 3 MM paper, dried and exposed to X-ray film (Kodak X-OMAT) for visualization of the shifted DNA protein complex. Gel retardation experiments were repeated at least three times for each template used during the course of this work. Reproducible results were obtained and the figures used in this dissertation (Results Section) contain representative data from a single experiment.

**DNase I footprinting analysis.** Sensitivity of RNA polymerase-promoter DNA complexes to DNase I was used to test for the structuring of DNA upstream of the promoter. DNase I footprinting analyses were carried out in 190-\( \mu \)l reaction mixtures containing 40 mM Tris-HCl (pH 8.0), 10 mM MgCl\(_2\), 50 mM NaCl, 1-10 \( \mu \)g *B. subtilis* RNA polymerase, and 10\(^5\) cpm of 5'-end labeled template DNA. Reaction mixtures were incubated at 37°C for 10 min. followed by the addition of 10 \( \mu \)l of 1:60 dilution of DNase I stock (2 mg/ml) for 30 seconds. After nuclease digestion, 19 \( \mu \)l of sodium acetate and 1 \( \mu \)l of *Saccharomyces cerevisiae* tRNA (1 \( \mu \)g/\( \mu \)l) were added to the reaction mixtures and then mixed with 350 \( \mu \)l of phenol/chloroform mixture (1:1) to stop
the digestion. Reactions were mixed using a vortex mixer and then placed on ice for 1 min followed by centrifugation for 2 min at 15,000 xg. The top aqueous layer containing the labeled DNA was recovered, ethanol precipitated, washed with 80% ethanol and vacuum dried. The dried pellet was suspended with 5 µl of formamide, 0.1% mg xylene cyanol, 0.1% mg bromphenol blue, and 10 mM ml 0.5 M EDTA (pH 7) prior to loading onto a 6% polyacrylamide (acrylamide:bis-acrylamide, 30:1.5) gel containing 7 M urea in TBE buffer. Samples containing DNA were denatured at 95°C for 5 min and held on ice prior to loading. Digests were electrophoresed at 1700 volts for 100 min and gels were dried before autoradiography using Dupont Cronex 4 film. Densitometry of autoradiograms was used to compare the intensities of DNA bands in the control and experimental lanes. The DNase I-generated bands were labeled in 10 base pair intervals relative to the transcription start site (+1) based on the direct comparison between the DNase I pattern and DNA sequencing reactions for the same template. DNase I footprinting experiments were repeated at least three times for each template tested during the course of this work. Reproducible results were obtained and the figures used in this dissertation (Results Section) contain representative data from a single experiment.

**In vitro transcription assay.** In the single-round, runoff transcription assay, DNA templates used were amplified using PCR from pUC8 plasmids containing the promoter. To ensure that equal amounts of DNA templates (i.e., the PCR products) were added in each transcription reaction, the DNA templates were separated on polyacrylamide gels and quantified using densitometry following ethidium bromide staining. Runoff transcription reactions were done in 20.0 µl volumes. Reactions contain DNA template, 0.5 µl *B. subtilis* RNA polymerase (1.5 mg/ml), 2 µl 10X transcription buffer (400 mM Tris-HCl, 100 mM MgCl₂, 500 mM NaCl), and water to reach a volume of 16.5 µl. After mixing reactions on ice, reactions were transferred to intended temperature for 5 min to start the reaction. 2 µl of nucleotides (20 mM CTP, 20 mM GTP, 4 mM UTP, 20 mM
ATP and 0.5 mCi/ml, 3000Ci/mmol [α-32P] UTP) and 1.5 µl of single-stranded M13 DNA (2.5 mg/ml) were added to each reaction simultaneously. The transcription reactions were incubated for 5 min and then quenched by adding urea to a final concentration of 5 M. Transcription products were electrophoresed at 500 volts for 1 hour through 6% denaturing polyacrylamide gel (acrylamide:bisacrylamide, 30:1.5, and 7 M urea) in TBE buffer. Dried gels were exposed to X-ray film (Kodak X-OMAT) and the incorporation of radioactivity into specific transcripts was quantified by densitometry.

In the multiple-round, runoff transcription assay, the same procedures mentioned in the single-round assay was followed with the exception that the single-stranded DNA was not added to the reactions. This allows the generation of multiple transcripts from the same promoter. After 10 min at the incubation temperature, all of the reactions were stopped by adding urea to a final concentration of 5M. Transcription products were analyzed as described above.

In order to detect stable closed promoter complexes, the single-round, runoff transcription assay was modified. Transcription reactions were made in duplicates. After the combination of water, buffer, template DNA and RNA polymerase on ice, the reactions were transferred to the incubation temperatures for 5 min. Nucleotides and single-stranded M13 DNA were added to each reaction simultaneously. After 10 seconds of the incubation with M13 DNA, one of the duplicates was shifted to 37°C and the other was kept at the initial incubation temperature. The non-shifted and temperature shifted reactions were incubated for 5 min and then stopped by adding urea. Transcripts were analyzed as described above. Single-round and multiple-round, runoff transcription assays were repeated at least three times for each template tested during the course of this study. Reproducible results were obtained and the figures used in this dissertation (Results Section) contain representative data from a single experiment.
Quantification of dissociated RNA polymerase-promoter complexes as detected by DNase I footprinting. Autoradiograms of DNase I-generated bands were quantified using densitometry. Three series of bands labeled "N", "O", and "T" were quantified. Regions not affected by RNA polymerase binding, "N" were used to normalize the intensity of the DNase I profiles among the lanes. The region "O" was protected from DNase I in open promoter complexes but not closed promoter complexes. Total complexes (open and closed promoter complexes) were identified as the series of bands marked "T." In order to measure the dissociation rate of these complexes on the autoradiogram, labeled regions were quantified and plotted as the fraction bound of open complexes or total complexes. To calculate the fraction bound as open promoter complexes, the following formula was used:

\[
\left( \frac{O_C}{N_C} - \frac{O_E}{N_E} \right) / \left( \frac{O_C}{N_C} \right)
\]

where:
- \(O_C\) is the series of bands quantified in region "O" for the control lane without RNA polymerase.
- \(N_C\) is the series of bands quantified in region "N" for the control lane without RNA polymerase.
- \(O_E\) is the series of bands quantified in region "O" in the presence of RNA polymerase.
- \(N_E\) is the series of bands quantified in region "N" in the presence of RNA polymerase.

To calculate the fraction bound as total complexes (open and closed promoter complexes), the following formula was used:

\[
\left( \frac{T_C}{N_C} - \frac{T_E}{N_E} \right) / \left( \frac{T_C}{N_C} \right)
\]
where:

- $T_C$ is the series of bands quantified in region "T" for the control lane without RNA polymerase.
- $N_C$ is the series of bands quantified in region "N" for the control lane without RNA polymerase.
- $T_E$ is the series of bands quantified in region "T" in the presence of RNA polymerase.
- $N_E$ is the series of bands quantified in region "N" in the presence of RNA polymerase.
RESULTS

This work investigates the role of intrinsic DNA curvature immediately upstream of the -35 promoter region in transcription initiation by *Bacillus subtilis* RNA polymerase, and the effect of the δ subunit on the stability of promoter-enzyme complexes. For this study, a promoter dependent on curved DNA for efficient utilization was chosen. This promoter, Alu156, is highly homologous to the consensus promoter sequence recognized by the major form of the *B. subtilis* RNA polymerase, the σ^A^-containing enzyme. Results of DNase I footprinting analyses with the Alu156 promoter showed that RNA polymerase wraps the DNA upstream of the promoter around the enzyme in a process greatly facilitated by curved DNA (Cheng, 1996).

**DNase I footprinting analysis of the *B. subtilis* RNA polymerase bound to Alu156 and AluExt promoters.** To confirm the finding that promoter DNA was wrapped around the *B. subtilis* RNA polymerase upon binding, the Alu156 and AluExt promoters were analyzed by DNase I footprinting under conditions that would permit RNA synthesis if the nucleoside triphosphates were present. The AluExt promoter was previously constructed by replacing the upstream curved DNA sequence of Alu156 with non-curved DNA (Figure 1). DNase I footprinting is well suited for this analysis since it can identify the site of protein binding, the footprint, and can detect the altered DNA conformation associated with DNA bending. DNA bent around a protein is protected from DNase I cutting on the surface facing the protein and is hypersensitive to DNase I cutting on the side facing outward. On autoradiograms, DNase I hypersensitive sites are identified as DNA bands darker than those in the control generated in the absence of the protein. Sites protected from DNase I cutting are observed as DNA bands lighter than the control. The banding pattern indicative of DNA wrapping is detected as a series of

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Figure 1: Nucleotide sequence of the Alu156 and AluExt promoters. For the Alu156 promoter (A) and the AluExt promoter lacking curved DNA (B), bases representing the +1 transcription start site, the -35 and the -10 regions are in bold type. The runs of adenines in the DNA upstream of the -35 region of Alu156 are underlined.
hypersensitive sites (dark bands) followed by protected sites (light bands) with a 10 base periodicity. Figure 2 corresponds to *B. subtilis* RNA polymerase bound to the Alu156 promoter as analyzed by the DNase I footprinting method. In Figure 2, each panel displays the DNase I digestion pattern of Alu156 in the absence or presence of RNA polymerase. In the samples where RNA polymerase was present, an extended area of protection was observed, typical of results with prokaryotic RNA polymerase. The DNase I patterns of the lower strand of Alu156 (Figure 2A) DNA show that the enzyme covers a region from about 50 nucleotides before and 20 after the start site for RNA synthesis. The nucleotides are numbered relative to the transcription start site at position (+1). Analysis of autoradiograms exhibited hypersensitive sites, represented by DNA bands that are more intense than the same band in the control lane, at -36 to -38, -48, -68 to -71, -80, -81, -91, -111 to -113, and -120. Short regions protected from DNase I attack were observed following these hypersensitive sites. A very pronounced hypersensitive site was observed at base -37. Additional DNase I hypersensitive sites were observed around -140 and further upstream. The (A+T)-rich region of curved DNA from -40 to -65 is weakly cleaved by DNase I in the control lane possibly due to the altered DNA conformation. This alteration reduces cleavage by DNase I and makes it more difficult to show enzyme-DNA interactions in this particular region. A densitometric scan of Figure 2A is shown in Appendix A.

A pattern of DNase I digestion consistent with RNA polymerase wrapping the DNA upstream of the -35 region around the enzyme was also observed on the upper strand of this promoter (Figure 2B). A large area of strong protection from DNase I was observed from +20 through -64. Sites of enhanced DNase I cleavage were found within this area of protection at -24 and -46. DNase I hypersensitive sites were observed at -65 to -67, -76 to -79, -97, -106, -107, -115 and -116. These sites were followed by protected regions. In agreement with the results from the lower strand, the RNA polymerase binding to the -10 and -35 regions were strongly protected from DNase I.
Figure 2: DNase I footprint analysis of the Alu156 promoter bound by RNA polymerase. Panel A corresponds the footprinting of the lower (template) strand and panel B contains the upper strand DNase I footprint. DNase I digestion patterns without (lanes with minus sign) and with (lanes with plus sign) RNA polymerase are presented. The bands are numbered relative to the transcription start site at position +1. This figure contains representative data from a single experiment. The experiment was repeated at least three times to insure reproducibility of the results.
attack, the curved DNA area from -40 to -65 was weakly cleaved by DNase I and hypersensitive sites were observed in roughly 10-11 base pair intervals. The 10-11 base pair repeat of alternating DNase I hypersensitive sites (enhanced cleavages) and sites protected from DNase I in both strands of Alu156 was indicative of DNA wrapping. A densitometric scan of Figure 2B is available in Appendix A.

The DNase I banding pattern for the promoter lacking the sequence-dependent DNA curvature, AluExt, was similar to that of the wild-type Alu156 promoter in the region from DNase I digestion from +20 to -46 in the lower strand (Figure 3A) and +20 to -42 in the upper strand (Figure 3B). DNase I hypersensitive sites were observed around the -48 region in both strands for this promoter. The dramatically hypersensitive site observed at -37 in the Alu156 promoter was greatly reduced in AluExt. Evidence of protein-DNA interactions consistent with DNA wrapping in the AluExt was not obvious. DNase I hypersensitive sites were observed at -47, -48, and -57 in the lower strand and at -44, -45, -47, -55, and -56 in the upper strand. Densitometric scans of Figures 3A and 3B are available in Appendix B. Figure 4 summarizes the DNase I footprinting results on both strands of Alu156 and AluExt promoters collected from several independent experiments. The RNA polymerase binding to the -10 and -35 regions observed as strong protection from DNase I digestion is found on both strands for each promoter. Extensive DNase I digestion pattern indicative of DNA wrapping was very obvious for Alu156. Evidence of protein-DNA interactions consistent with DNA wrapping was less obvious for AluExt promoter which lacks the curved DNA. A periodicity of approximately 10 base pairs in the pattern of enhanced cleavages and sites protected from DNase I upstream the -35 regions observed for each strand of the Alu156 promoter. This repeat on the lower strand is offset in relation to the upper strand by about 2 to 3 base pairs. This offset on the primary sequence places these sites on the same face of the B-form DNA helix. This pattern provides strong evidence for DNA wrapping around the RNA polymerase.
Figure 3: DNase I footprint analysis of RNA polymerase binding to AluExt promoter lacking curved DNA. Panel A corresponds the footprinting of the lower (template) strand DNA footprint, and panel B represents the upper strand DNA footprint. DNase I digestion patterns without (lanes with minus sign) and with (lanes with plus sign) RNA polymerase (6 μg) are presented. The bands are numbered relative to the transcription start site at position +1. This figure contains representative data from a single experiment. The experiment was repeated at least three times to insure reproducibility of the results.
Figure 4: The summary of DNase I footprinting results on both DNA strands for the Alu156 and the AluExt promoters. The summary of DNase I footprinting results on both DNA strands for the Alu156 (A) and the AluExt (B) promoters. The nucleotide sequence of both promoters is shown with the +1 transcription start site and the -35 and -10 regions in bold type. The lines over the upper strands and below the lower strands indicate regions where the bound *Bacillus subtilis* RNA polymerase protected the DNA from DNase I cleavage. The arrows mark sites hypersensitive to DNase I digestion. The results were compiled from three separate experiments.
It should be noted that the possibility of a second RNA polymerase binding to the upstream DNA sequence was excluded by footprinting the promoters at various RNA polymerase concentrations. At the lowest concentration of RNA polymerase where a footprint was observed (i.e., RNA polymerase was bound to the promoter), DNA wrapping was present. The DNase I digestion pattern attributed to DNA wrapping disappeared at the same concentration as the main footprint. This indicates that wrapping was not the result of binding another RNA polymerase to upstream sequence.

**Effects of upstream synthetic curved DNA sequence on promoter function.** Since it was shown that one naturally occurring curved DNA sequence could functionally replace another on the Alu156 promoter, enhanced promoter function was attributed to the curved DNA (McAllister and Achberger, 1988). However, there may be other upstream DNA sequences essential for enhanced promoter function. To test this, synthetic curved DNA sequences were used to replace the native sequence of Alu156. In addition, the effect of the number of adenine tracts upstream of the -35 region on promoter function was tested. Therefore, a series of altered promoters were constructed. In these constructed promoters, the native curved DNA was exchanged for synthetic DNA containing one, two, or three adenine tracts (Alu1A, Alu2A, and Alu3A) phased with the helical repeat of the B-form DNA. Figure 5 represents nucleotide sequence of Alu156, AluExt, and promoters with one, two, or three adenine tracts (A-tracts). To explore the effect of synthetic curved DNA immediately upstream of the -35 region on RNA polymerase binding, gel shift studies were performed. In these experiments, various concentrations of RNA polymerase were added to each of the promoter constructs and unbound DNA (free) was separated from the enzyme bound complexes (bound) by electrophoresis on polyacrylamide gels (Figure 6). No RNA polymerase was added to lanes labeled with a minus sign. The results demonstrated that, for each concentration of RNA polymerase used, the DNA fragment containing upstream DNA curvature (Alu156) was most efficiently bound by the RNA polymerase. In contrast,
Figure 5: Nucleotide sequence immediately upstream of -20 for the Alu156 promoter and derivatives with various numbers of adenine tracts. Promoters with one (Alu1A), two (Alu2A), or three (Alu3A) adenine tracts phased with the helical repeat of the B-form DNA are shown with the adenine tracts are underlined. The -35 region is in bold type.
Figure 6: Gel retardation analysis of Alu156, AluExt, and promoters with synthetic adenine tracts. Equivalent amounts of $^{32}$P end-labeled DNA fragments were incubated with different concentrations of RNA polymerase at 37°C for 10 minutes, followed by electrophoresis. The location of unbound DNA (Free) and enzyme bound complexes are indicated in Panel A. No RNA polymerase was added to lanes labeled with a minus sign. The RNA polymerase concentration (1.2, 0.6, 0.3 μg/15 μl) was decreased as it is marked by the symbol. RNA polymerase binding observed in panel A was quantified and plotted in panel B as the fraction bound relative to the RNA concentration. Results for the Alu156 (●), AluExt (■), Alu1A (▲), Alu2A (▼), and Alu3A (♦) promoters are presented. This figure contains representative data from one of three repeats of this experiment.
promoter demonstrated reduced RNA polymerase binding. Alu1A had a binding affinity similar to AluExt, and the binding increased as the number of A-tracts were added to the constructed promoters. This suggests that RNA polymerase binding is enhanced by the increase in the number of adenine tracts (i.e., the degree of curvature) added immediately upstream of the -35 region of the constructed promoters. The Alu3A promoter exhibited at least 75% of the binding observed for Alu156. Since the relative affinity of Alu3A was not equal to Alu156, there may be additional sequence elements contributing to RNA polymerase binding or the synthetic DNA may not properly aligned.

To further characterize the effect of synthetic curved DNA sequence on DNA-RNA polymerase interactions, DNase I footprinting of B. subtilis RNA polymerase bound to promoters Alu156, AluExt, Alu1A, Alu2A, and Alu3A was performed. Each promoter sequence was treated with DNase I in the absence and presence of RNA polymerase. Figure 7A is a comparison of the DNase I digestion patterns for the upper DNA strands of the Alu156 and Alu3A promoters. Alu156, which contains the native curved DNA, and Alu3A, which has a synthetic curved DNA region comparable in length to that in Alu156, both show a region of interrupted protection from the +15 region to the curved DNA region starting at -40. Interestingly, the Alu3A promoter is less protected from DNase I in the -15 region of the promoter relative to Alu156. This may reflect the difference in binding observed in gel shift experiments. Each promoter displayed the wrapping of DNA around the RNA polymerase. This distinctive pattern extended through the curved DNA and upstream to at least -120. Based on repeated DNase I footprinting assays, it was concluded that while Alu3A was wrapped by the RNA polymerase, the wrapping was less pronounced than that observed with Alu156. Densitometric scans of the lanes in Figure 7A are shown in Appendix C.

The DNase I footprinting analysis of the Alu1A and Alu2A promoters demonstrated little if any evidence for the wrapping of DNA upstream of the promoter around the RNA polymerase (Figure 7B). The footprints for all promoters share striking
Figure 7: DNase I footprinting of RNA polymerase bound to Alu156, AluExt, Alu1A, Alu2A, and Alu3A promoters. DNA fragments were incubated in the absence (lanes with a minus sign) or in the presence (lanes with a plus sign) of 6 µg RNA polymerase at 37°C for 10 minutes. Numbers indicate positions on the DNA sequence with respect to the transcription start site (+1) on the upper (nontemplate) strand. Panel A represents the DNase I footprinting of RNA polymerase bound to Alu156 and Alu3A. Panel B represents the DNase I footprinting of RNA polymerase bound to Alu156, AluExt, Alu1A, and Alu2A promoters. This figure contains representative data from a single experiment. The experiment was repeated at least three times to insure reproducibility of the results.
similarity in the strongly protected area between +15 to -40 with localized gaps in the protection region. Upstream of this region, for the original promoter, Alu156 with curved DNA, a pattern of DNase I hypersensitive sites and sites protected from cutting repeated in phase with the helical periodicity of the DNA was observed. This pattern was indicative of DNA wrapped around the RNA polymerase. Evidence for the wrapping of upstream DNA sequences was greatly diminished when the curved DNA was removed and replaced with non-curved fragment (AluExt). Promoters with one or two A-tracts displayed little evidence of DNA wrapping. Based on densitometric scans of Figure 7B (Appendix D), the Alu2A promoter displayed weak evidence of DNA wrapping relative to AluExt and Alu1A. Increased DNA wrapping correlated with the number of adenine tracts added.

Several studies were done to measure the strength of promoters listed in Figure 5 using single-round and multiple-round, runoff transcription assays. Promoter strength was defined as the relative number of RNA transcripts initiated from a promoter per unit time. The purpose of these experiments was to determine if the synthetic curved DNA upstream of the -35 region affects overall promoter strength \textit{in vitro}. In the single-round runoff transcription assay, the number of transcribable complexes at the time of transcription initiation was measured. The multiple-round transcription assay measured the number of transcribable complexes that formed per unit time. The two assays are very similar except that in the single-round transcription assay, single-stranded M13 DNA is added 10 seconds after the addition of the initiated nucleotides. Single-stranded DNA is a strong competitor that binds free RNA polymerase very quickly and prevents the rebinding of free RNA polymerase to promoter containing DNA once the enzyme has been released. Therefore, multiple rounds of transcription are prevented. All RNA polymerase-promoter complexes, other than the open promoter complexes, are relatively unstable, and the majority dissociate prior to initiation. Therefore, RNA polymerase released by these less stable complexes, binds the single-stranded competitor DNA and is
removed from consideration. The $^{[32P]}$ end-labeled transcripts were quantified by densitometry of the autoradiograms of polyacrylamide gels. Results of single-round and multiple-round, runoff transcription assay are presented in Figure 8. The data were normalized relative to the Alu156 promoter to permit direct comparison. Typically there is five fold more transcripts for the multiple-round transcription assay relative to the single-round transcription assay. The highest levels of transcripts were observed with the original Alu156 promoter. AluExt promoter which lacks the curved DNA showed the lowest number of transcripts. The strength of promoter with one A-tract was similar to AluExt where the fewest transcripts were formed. The Alu2A promoter was stronger than Alu1A, and Alu3A promoter demonstrated more transcripts than Alu2A. In general, the more A-tracks present on the promoter, the greater the level of transcription.

The results of gel retardation experiments, DNase I footprinting studies, and runoff transcription assays demonstrate the contribution of synthetic DNA upstream of the -35 region to promoter function and suggest that the degree of curvature affects promoter binding and transcription initiation.

Alignment of the curvature in Alu3A promoter. The correct orientation of the curved DNA relative to the -10 and -35 regions is critical for efficient promoter function (McAllister and Achberger, 1989). When the curved DNA was not positioned in the same orientation as the original Alu156, binding to RNA polymerase was reduced, DNase I digestion pattern indicative of DNA wrapping was less obvious (Cheng, 1996) and efficiency of transcription was decreased (McAllister and Achberger, 1989). Based on gel retardation analysis, DNase I footprinting experiments, and runoff transcription assays, it was obvious that the Alu3A promoter was not as efficient as the Alu156 promoter. Based on sequence comparison between the Alu156 and Alu3A promoters, two possibilities were addressed in an attempt to explain why Alu3A was not as strong as Alu156. Since proper alignment of the curved DNA to the promoter is important for
Figure 8: Transcribable promoter complexes formed by Alu156 and promoters with various numbers of adenine tracts at 37°C. Relative number of transcripts for Alu156, AluExt, Alu1A, Alu2A, and Alu3A were determined by densitometry from an autoradiograph following polyacrylamide gel electrophoresis of [\(^{32}\)P] end-labeled transcripts. This figure contains representative data for the single round run-off transcription assay (open bars) in the presence of M13 DNA or multiple rounds run-off transcription assay (filled bars) without M13 DNA addition. This experiment was repeated at least three times to ensure reproducibility of the results. The number of transcripts were normalized relative to the Alu156 promoter for each assay.
efficient promoter function (McAllister and Achberger, 1989), the orientation of the curved DNA in Alu3A may not have been optimal compared with Alu156. An alternate explanation is that there are sequences within the curved DNA sequences that affect promoter function. To test for the first possibility, two Alu3A promoter derivatives were constructed and named Alu3A(+1) and Alu3A(-1). Alu3A(+1) has an additional thymine between the -35 region and the first run of adenine tract, whereas a cytocine has been deleted from Alu3A at -37 forming Alu3A(-1) promoter (Figure 9). The insertion or deletion of a single base pair changes the rotational orientation of the curve by about 34° relative to the -10 and -35 regions of the promoter. When PCR amplified DNA fragments containing the wild-type Alu156, Alu3A, and its derivatives were electrophoresed through a non-denaturating polyacrylamide gel at room temperature, their migration rates indicated differences in the shapes of the DNA molecules (Figure 10). A striking feature of curved DNA is its abnormal electrophoretic mobility on acrylamide gels when electrophoresed under non-denaturating conditions. In general, the presence of a curved DNA retards the electrophoretic mobility of DNA fragments. The addition of one base decreased the apparent size of Alu3A(+1) by 20 base pairs relative to Alu3A. The deletion of one base pair increased the apparent size of Alu3A(-1) by 10 base pairs relative to Alu3A (Table 1). As documented for the Alu156 promoter (McAllister and Achberger, 1989), this indicates that there are actually two curves in the Alu3A DNA separated by the nucleotide insertion/deletion site or that this site is located within the curved sequence. As the upstream curve is rotated relative to the downstream curve due to nucleotide insertion to make Alu3A(+1), this promoter DNA was more S-shaped relative to Alu3A, and migrated faster than a U-shaped molecule. For Alu3A(-1) promoter, the promoter DNA was more U-shaped relative to Alu3A, and migrated more slowly.
Figure 9: Nucleotide sequence immediately upstream of -20 for the Alu156, Alu3A, Alu3A(+1), and Alu3A(-1) promoters. Adenine tracts associated with curved DNA are underlined. The -35 region is in bold type.
Figure 10: Electrophoretic mobility of Alu156, Alu3A, and derivatives of Alu3A. The electrophoresis was performed on a non-denaturing 10% polyacrylamide gel at room temperature. Size in base pairs of selected bands is indicated for the DNA size marker (M), pBR322 DNA digested with MspI.
Table 1: Comparison of the Apparent Length of Promoter Containing DNA Fragment Determined by Polyacrylamide Gel Electrophoresis at 25°C.

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Actual Length (a)</th>
<th>Apparent Length (b)</th>
<th>Apparent/Actual</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alu156</td>
<td>296</td>
<td>338</td>
<td>1.14</td>
</tr>
<tr>
<td>Alu3A</td>
<td>327</td>
<td>375</td>
<td>1.15</td>
</tr>
<tr>
<td>Alu3A(+1)</td>
<td>328</td>
<td>355</td>
<td>1.08</td>
</tr>
<tr>
<td>Alu3A(-1)</td>
<td>326</td>
<td>385</td>
<td>1.17</td>
</tr>
</tbody>
</table>

\(a\) As determined by nucleotide sequencing of the DNA fragment.

\(b\) As determined by polyacrylamide gel electrophoresis at 25°C.

Gel retardation analysis was performed to evaluate the affinity of RNA polymerase for the original Alu156 and Alu3A and its derivatives (Figure 11). RNA polymerase was most efficiently bound to Alu156. Alu3A(+1) was the least effectively bound promoter, while Alu3A(-1) was comparable to Alu3A in binding RNA polymerase. This indicates that if the synthetic curved DNA sequence in Alu3A was not optimally aligned with the promoter, it was close to the optimal orientation.

DNase I footprinting using RNA polymerase core plus sigma was used to analyze promoter-enzyme interactions. The basic footprint covering the -10 and -35 regions observed for Alu3A and its derivatives was very similar to that for Alu156. DNase I cleavage patterns indicative of DNA wrapping were more evident for Alu156 than Alu3A (Figures 12 and 13). Figures 14 and 15 represent densitometric scans of the DNase I footprinting analyses obtained for Alu3A(-1) and Alu3A(+1) promoters, respectively. The Alu3A(+1) promoter exhibited strong evidence of wrapping within the curved DNA (i.e., the -40 to -70 region), but lacked several key hypersensitive sites, such as those at the -75 region (Figure 14). The Alu3A(-1) displayed the strongest evidence for the wrapping of upstream DNA about the RNA polymerase (Figure 15).
Figure 11: Gel retardation analysis of Alu156, Alu3A, and derivatives of Alu3A. Equivalent amounts of $^{32}$P end-labeled DNA fragments were incubated with various amounts of RNA polymerase saturated with δ subunit at 37°C for 10 minutes, followed by electrophoresis on a non-denaturing gel at room temperature. Lanes marked with a minus (Panel A) represent the absence of RNA polymerase. The DNA bands representing the RNA polymerase bound complexes and free DNA are marked. The RNA polymerase concentration (1.2, 0.6, 0.3 μg/15 μl) was decreased as it is marked by the symbol. RNA polymerase binding observed in panel A was quantified and plotted in panel B as fraction bound relative to RNA concentration. Results for the Alu156 (●), Alu3A (■), Alu3A+1 (▲), and Alu3A-1 (▼) promoters are presented. This figure contains representative results from one of three repeats of this experiment.
Figure 12: Densitometric scans of the DNase I footprinting analyses of the Alu156 promoter. Panel A and B represent the densitometric analysis of the DNase I digestion pattern obtained for the Alu156 promoter in the absence (panel A) or presence (panel B) of 1.2 μg/μl RNA polymerase. In panel C, the two densitometric scans have been superimposed to highlight regions of protection from DNase I and enhanced DNase I cleavage. The scans are listed in 10 base pair intervals to approximate the location of DNA bands relative to the transcription start site (+1).
Figure 13: Densitometric scans of the DNase I footprinting analyses of the Alu3A promoter. Panel A and B represent the densitometric analysis of the DNase I digestion pattern obtained for the Alu3A promoter in the absence (panel A) or presence (panel B) of 1.2 μg/μl RNA polymerase. In panel C, the two densitometric scans have been superimposed to highlight regions of protection from DNase I and enhanced DNase I cleavage. The scans are listed in 10 base pair intervals to approximate the location of DNA bands relative to the transcription start site (+1).
Figure 14: Densitometric scans of the DNase I footprinting analyses of the Alu3A(+1) promoter. Panel A and B represent the densitometric analysis of the DNase I digestion pattern obtained for the Alu3A(+1) promoter in the absence (panel A) or presence (panel B) of 1.2 µg/µl RNA polymerase. In panel C, the two densitometric scans have been superimposed to highlight regions of protection from DNase I and enhanced DNase I cleavage. The scans are listed in 10 base pair intervals to approximate the location of DNA bands relative to the transcription start site (+1).
Figure 15: Densitometric scans of the DNase I footprinting analyses of the Alu3A(-1) promoter. Panel A and B represent the densitometric analysis of the DNase I digestion pattern obtained for the Alu3A(-1) promoter in the absence (panel A) or presence (panel B) of 1.2 µg/µl RNA polymerase. In panel C, the two densitometric scans have been superimposed to highlight regions of protection from DNase I and enhanced DNase I cleavage. The scans are listed in 10 base pair intervals to approximate the location of DNA bands relative to the transcription start site (+1).
While hypersensitive sites upstream of the -35 region of Alu3A(-1) agree well with Alu156, the regions of protection were more difficult to identify. The Alu156 promoter and Alu3A series have different DNA sequences upstream of the curved DNA making direct comparisons difficult. The summary of densitometric scans for Alu156, and Alu3A and derivatives of Alu3A is shown in Figure 16. These data suggest that the original orientation of the curved DNA sequence in Alu3A promoter was not far from optimal for promoter function. It is possible that the sequence within the curved DNA region contributes to promoter function by interacting with the RNA polymerase.

Effect of (A+T)-rich region on promoter function. The second possibility for why Alu3A was not as strong as Alu156 was that there are sequences within the curved sequences that affect binding. Some highly expressed promoters contain an (A+T)-rich promoter element, the so called "UP element," located upstream of the -35 region. The UP element enhances RNA polymerase binding, interacts directly with the α subunits of RNA polymerase, and stimulates transcription both in vivo and in vitro (Fredrick and Helmann, 1997; Fredrick et al., 1995; Ross et al., 1993). Since the curved DNA region is an (A+T)-rich sequence and it has been documented that the C-terminal domain of the α subunit of the RNA polymerase binds to (A+T)-rich regions, the contribution of an AT content of curved DNA was investigated. Derivatives of the Alu156 promoter in which the DNA curvature immediately upstream of the -35 region was altered or exchanged for non-curved DNA rich in (A+T) were constructed. In one promoter, AluUnc, a thymine residue was inserted in the middle of each A-tract upstream of Alu156. A thymine in an A-tract greatly diminishes the curvature while preserving the A-T rich nature of this promoter (Jazbi, 1997). When PCR amplified DNA fragments containing the original Alu156 promoter and AluUnc were electrophoresed through a non-denaturating polyacrylamide gel at room temperature, their migration rates were different even though they were the same size (Figure 17). The difference in the migration rate of AluUnc relative to Alu156 indicates that the DNA curvature of AluUnc was significantly reduced.
Figure 16: The summary of densitometric scans of the DNase I footprints of Alu156 and Alu3A and derivatives of Alu3A. Panels A, B, C, and D represent the densitometric analyses of the DNase I footprints obtained for the Alu156, Alu3A, Alu3A(+1), and Alu3A(-1) promoters, respectively. In all panels, the two densitometric scans have been superimposed to highlight regions of protection and enhanced cleavage. The scans are listed in 10 base pair intervals to approximate the location of DNA bands relative to the transcription start site (+1).
Figure 17: Electrophoretic mobility of the Alu156 and AluUnc promoters. Gel electrophoresis was performed on a non-denaturing 10% polyacrylamide gel at room temperature. Sizes (bp) of the bands of the DNA size standard (M), pBR322 digested with AvoI and AvoI/EcoRI, are indicated.
In a second promoter, AluUp, DNA sequences upstream of -35 region in Alu156 were replaced by the α binding site (UP element) of *E. coli* ribosomal RNA promoter *rrnBpJ*. DNA sequences of AluUnc and AluUp are listed in Figure 18 in comparison with nucleotide sequences of Alu156 and AluExt. Gel retardation analysis was performed to compare the binding of *B. subtilis* RNA polymerase to the collection of promoters listed in Figure 18. Figure 19 is a representative autoradiogram for the gel retardation analysis. For each concentration of RNA polymerase used, the enzyme displayed the highest affinity for the original Alu156. AluUnc and AluUp promoters showed a relatively high affinity for RNA polymerase when compared to AluExt which displayed the lowest affinity to RNA polymerase. Typically, AluUp and AluUnc displayed about 60% of the binding observed for Alu156. This suggests that the (A+T)-rich component of curved DNA was important for RNA polymerase binding but also that the DNA curvature itself enhanced RNA polymerase binding.

To investigate the specific interactions between *B. subtilis* RNA polymerase and the (A+T)-rich promoters, DNase I footprinting analysis was performed. This addresses the role of (A+T)-rich region upstream of the -35 region in DNA structuring. Figure 20 represents DNase I banding pattern for the lower strands of the tested promoters. The DNase I footprinting pattern for all promoters was similar to that of the original Alu156 in the region protected from DNase I cleavage from +18 to -55. DNase I cleavage pattern indicative of wrapping was greatly reduced in AluUnc and AluUp in comparison to Alu156. For the AluExt promoter, evidence of the wrapped RNA polymerase complex was not obvious. The AluUp promoter displayed a strongly protected region between -38 and -58 where the (A+T)-rich sequence (UP element) is located. Caution should be exercised when interpreting this result since AluExt, which lacks the (A+T)-rich region exhibit some protection in the same region. Densitometric scans of the DNase I patterns displayed in Figure 20 are available in Appendix E. In general, the data...
Figure 18: Nucleotide sequence of the Alu156, AluExt, AluUnc, and AluUp promoters. (A+T)-rich regions upstream of the -35 region are underlined. The -10, -35 and +1 regions are in bold type. An asterisk was placed under the bases in AluUnc that differ from Alu156.
Figure 19: Gel retardation analysis of Alu156, AluExt, AluUnc, and AluUp promoters. Equivalent amounts of $^{32}$P end-labeled DNA fragments were mixed with various amounts of RNA polymerase at 37°C for 10 minutes, followed by electrophoresis. Lanes marked with a minus sign (Panel A) received no RNA polymerase. The DNA bands representing the RNA polymerase bound complexes and free DNA are marked. The RNA polymerase concentration (1.2, 0.6, 0.3 µg/15 µl) was decreased as represented by the symbol. RNA polymerase binding observed in panel A was quantified and plotted in panel B as the fraction bound relative to the RNA polymerase concentration. Results for the Alu156 (●), AluExt (■), AluUnc (▲), and AluUp (▼) promoters are presented. This figure contains representative results from one of three repeats of this experiment.
Figure 20: DNase I footprinting analysis of the Alu156, AluExt, AluUnc, and AluUp promoters. DNase I cleavage patterns generated in the presence and absence of 6 µg RNA polymerase are indicated by plus and minus signs. The DNA bands are numbered relative to the transcription start site at position +1 on the lower (template) strand. This figure contains representative data from a single experiment. The experiment was repeated at least three times to insure reproducibility of the results.
demonstrate the importance of the DNA curvature in stabilizing the wrapped intermediate observed by DNase I footprinting.

The RNA polymerase-promoter complex in which the DNA is wrapped around the enzyme is an unstable closed promoter complex. The *B. subtilis* RNA polymerase-promoter complex that displays the features of DNA wrapping was tentatively identified as a closed promoter complex (Cheng, 1996). To verify the presence of these closed complexes and to investigate their stability, RNA polymerase-Alu156 promoter complexes were challenged with single-stranded M13 DNA for 10 and 30 sec prior to DNase I footprinting analysis. Single-stranded DNA (ssDNA) is a strong DNA competitor that quickly binds free RNA polymerase, but not to enzyme that is stably bound to DNA, or enzyme already in the process of RNA synthesis (Walter *et al.*, 1967). Sensitive closed complexes rapidly and irreversibly dissociate upon ssDNA challenge. The Alu156 promoter was footprinted without and with ssDNA at 37°C and 0°C (Figures 21 and 22, receptively). The Alu156 promoter bound by RNA polymerase displayed a DNase I pattern upstream of the -35 region indicative of DNA wrapping, and the footprint region spanned from +18 to -50 (Figure 21). Upon addition of ssDNA, a minor decrease in bound complexes was observed at 37°C. However, the DNase I pattern attributed to DNA wrapping was reduced significantly when ssDNA was added for 10 seconds prior to DNase I and essentially eliminated when added 30 second prior to DNase I. This is consistent with the loss of the RNA polymerase complexes in which the DNA is wrapped around the enzyme. At 0°C, where open complexes are not detected, the RNA polymerase footprint covered +1 to -50, which is typical for closed promoter complexes (Figure 22). At this temperature, the loss of the distinctive DNase I pattern upstream of the curved DNA was accompanied by a significant loss of bound complexes upon the addition of ssDNA. Residual binding identifies a closed promoter complex that is stable in the presence of ssDNA and lacks the DNase I pattern indicative
Figure 21: DNase I footprinting of RNA polymerase bound to the Alu156 promoter in the presence of single-stranded M13 DNA at 37°C. DNA fragments were incubated in the absence (lanes with a minus sign) or in the presence (lanes with a plus sign) of 6 µg RNA polymerase for 10 minutes. The lane marked with a plus sign shows binding of RNA polymerase without single-stranded M13 DNA. The lanes marked with "10 s" and "30 s" RNA polymerase was present but received single-stranded M13 DNA for 10 seconds and 30 seconds prior to DNase I treatment. Numbers at the side at the indicated positions on the DNA sequence with respect to the transcription start site (+1) on the lower (template) strand for Alu156. This figure contains representative data from a single experiment. The experiment was repeated at least three times to insure reproducibility of the results.
Figure 22: DNase I footprinting of RNA polymerase bound to the Alu156 promoter in the presence of single-stranded M13 DNA at 0°C. DNA fragments were incubated in the absence (lanes with a minus sign) or in the presence of 6 μg RNA polymerase for 10 minutes. The lane marked "30 s" received single-stranded M13 DNA 30 seconds prior to DNase I treatment. The lane marked with a plus sign did not receive single-stranded M13 DNA. Numbers at the side at the indicated positions on the DNA sequence with respect to the transcription start site (+1) on the lower (template) strand for Alu156. This figure contains representative data from a single experiment. The experiment was repeated at least three times to insure reproducibility of the results.
of DNA wrapping (CPC₈). Densitometric scans of Figures 21 and 22 are available at Appendixes F and G, respectively. Since the closed promoter complex exhibiting DNA wrapping (CPC₆) quickly dissociated in the presence of ssDNA, it is less stable than the CPC₈ and would represent an earlier intermediate in the kinetic pathway leading to transcription initiation.

**Evidence for the kinetic intermediate (CPC₈) at the level of transcription.** A single-round, runoff transcription assay was designed to quantitate closed promoter complexes that survive the ssDNA challenge (CPC₈). Single-round, runoff transcription was measured as a function of incubation temperature to take advantage of the lack of detectable open complexes at 0°C. Briefly, the assay was carried out by first incubating the RNA polymerase with equal amounts of the tested promoter at 0, 10, 20, and 30°C. An excess of single-stranded M13 DNA and a mixture of all four nucleoside triphosphates including [α-³²P] ATP were then added to each reaction. In the presence of ssDNA, unstable complexes, including CPC₆, quickly dissociated and open complexes, if present, initiate transcription in the presence of the nucleotide triphosphates. The first set of reactions were stopped after five minutes at the incubation temperature. The second set of reactions were held at the initial temperature for 10 seconds after the addition of ssDNA with the nucleotide mixture and then transferred to 37°C for 5 minutes prior to stopping the reactions. This temperature shift allowed stable complexes that survived the ssDNA challenge to form open complexes and start transcription. The samples were then analyzed by polyacrylamide gel electrophoresis under denaturing conditions (i.e., gels with 7 M urea electrophoresed at 55°C). To test the effect of curved DNA and (A+T)-rich DNA on the formation of CPC₈ and transcription initiation, the promoter series Alu156, AluUnc, AluUp, and AluExt were used (Figure 23). Figure 23, panel A demonstrates the relative number of transcripts observed in a single-round at each temperature. As expected, no transcripts were
Figure 23: Temperature-shift transcription assay to detect stable closed complexes in the absence of the δ subunit. Transcribable promoter complexes were measured in a single-round, runoff transcription assay as a function of temperature. Representative data for the relative number of transcripts for Alu156 (○), AluExt (■), AluUnc (▼), and AluUp (▲) were determined by densitometry of an autoradiograph of a polyacrylamide gel containing [32P] labeled transcripts. The assay was conducted without (panel A) or with (panel B) shifting the reactions to 37°C to permit stable closed promoter complexes to form open promoter complexes and initiate transcription. This figure contains representative data from one of several repeats of this experiment.
produced at 0°C. The number of transcripts increased with temperature as open promoter complexes were formed.

In agreement with previous RNA polymerase binding studies, Alu156 was the most effective, and AluExt was the least effective promoter. Panel A was used to demonstrate the level of transcription possible at 0°C and 10°C. Panel B shows the level of transcription observed when RNA polymerase-promoter complexes formed at the listed temperature were shifted to 37°C ten seconds after the addition of ssDNA and nucleoside triphosphates. The most obvious finding was the large number of transcripts observed for Alu156 at 0°C and 10°C. Since the temperature shift permits closed complexes that survived ssDNA challenge to form transcripts, the difference in transcription between panel A and panel B at 0°C represent the CPCS described above. The curved DNA of Alu156 permitted the formation of more CPCS than did the (A+T)-rich regions of AluUnc and AluUp. This demonstrates the significance of curved DNA in stimulating the formation of ssDNA stable closed complexes that will eventually isomerize into open complexes and initiate transcription.

Effect of subunit composition of \textit{B. subtilis} RNA polymerase on promoter-enzyme interactions. The presence of the \( \delta \) subunit on the \textit{B. subtilis} RNA polymerase is known to weaken the interaction between the enzyme and the DNA leading to increased efficiency in promoter selection (Achberger and Whiteley, 1981; Achberger \textit{et al.}, 1982; Dobinson and Spiegelman, 1987; Juang and Helmann, 1994; 1995). Since the removal of \( \delta \) from RNA polymerase greatly stabilizes enzyme DNA interactions, all DNase I footprinting analyses in this study were performed with this form of RNA polymerase (Eo). To examine the effect of \( \delta \) on the enzyme-promoter complexes detected by DNase I footprinting, the Alu156 promoter was analyzed with and without saturating amounts of the \( \delta \) subunit (Figure 24). In the absence of \( \delta \), the DNase I pattern observed for Eo included the strongly protected footprint from +18 to the -55 region and the upstream
Figure 24: DNase I footprinting of RNA polymerase bound to the Alu156 promoter in the presence of the δ subunit. DNA fragments were incubated in the absence (lane with a minus sign) or in the presence of 6 μg RNA polymerase for 10 minutes. The δ subunit was mixed with the core enzyme before incubation with Alu156 promoter. The lanes marked "0.5" and "1.0" received 0.03 μg/μl and 0.06 μg/μl δ subunit, which represent one-half the amount (0.5) and the full amount (1) of δ needed to saturate the RNA polymerase. Numbers at the side at the indicated positions on the DNA sequence with respect to the transcription start site (+1) on the lower (template) strand for Alu156. This figure contains representative data from a single experiment. The experiment was repeated at least three times to insure reproducibility of the results.
pattern of DNase I hypersensitive sites followed by protection from DNase I that indicate the presence of DNA wrapped around the enzyme. With the addition of saturating amounts of δ to Eσ, two general changes were observed in the DNase I pattern for Alu156. First, the DNase I pattern indicative of DNA wrapping was lost. Second, the protection in the footprint area from +18 to -55 region was reduced (+1 to -55) due to a loss of some of the RNA polymerase bound complexes, and the conversion of some of the open promoter complexes to closed promoter complexes was noted with loss of protection in the +18 to +1 region. (i.e., typically, DNase I footprints for closed promoter complexes do not extend beyond the +1 region.) When less than saturating amounts of δ subunit were added to Eσ, the same effects were evident, but clearly intermediate in intensity. Densitometric scans of these data are available in Appendix H. The results observed with Eσ plus δ are consistent with weaker RNA polymerase-promoter interactions. Specifically, the loss of protection in the +18 to +1 region indicated weaker binding of RNA polymerase to the DNA. The loss of the DNase I pattern indicating DNA wrapping suggested that, in the presence of δ, the CPCW was too unstable to be footprinted. Addition of δ to RNA polymerase at 0°C resulted in the loss of the CPCW but not the CPCs, in which the DNA is not wrapped around RNA polymerase (data not shown).

To confirm that the δ subunit weakened the interactions between RNA polymerase and the Alu156 promoter, the dissociation of the enzyme was measured using the filter binding assay. RNA polymerase was allowed to bind [32P]-labeled DNA containing the Alu156 promoter in the presence or absence of δ. An excess of ssDNA was added to each reaction and RNA polymerase-promoter complexes were collected on nitrocellulose filters as a function of time after the ssDNA addition. In the absence of δ, there was a decay curve typical for a mixture of enzyme-DNA complexes with different half lives (Figure 25). The addition of δ decreased the stability of the enzyme-DNA
Figure 25: Effect of the δ subunit on the dissociation of RNA polymerase from the Alu156 promoter. The stability of RNA polymerase-DNA complexes formed with (●) or without (■) the delta subunit were measured in the presence of 7.5 μg single-stranded M13 DNA. The nitrocellulose filter retention of 8.4 μg of RNA polymerase bound to [32P] end-labeled DNA fragments containing Alu156 was determined. The original Alu156 was measured 10, 30, 90, 270, and 810 seconds after adding M13 DNA. This figure contains representative data from one experiment that has been repeated at least three times.
complexes as seen by the increased rate of dissociation, and based on the amount of DNA bound in the final plateau value, δ reduced the number of stable complexes formed. Both effects, the increased dissociation rates and reduced number of stable complexes, coincide with the effect of δ on the DNase I footprints of Alu156. The ability of δ to reduce the stability of RNA polymerase-DNA interactions explains why the easily dissociated CPC\textsubscript{W} was not observed in DNase I footprinting analyses in the presence of δ (Figure 24). In DNase I footprinting analyses, the addition of δ made the CPC\textsubscript{W}, the closed promoter complex with the DNA wrapped around the enzyme, undetectable.

To demonstrate that the failure to detect CPC\textsubscript{W} was due to the reduced stability of complexes in the presence of δ, the DNase I footprinting assay was used to monitor the rate of complex dissociation. Since the DNase I treatment required 30 seconds, dissociation times should be interpreted as the time listed plus the treatment time. Figure 26 demonstrates the difference in the stability of complexes formed by Eσ and Eσ plus δ. In the absence of δ, a typical footprint is observed from +18 to the -55 region, and the presence of DNase I hypersensitive sites and sites protected from DNase I with a ten base pair periodicity indicated the wrapping of DNA around the RNA polymerase. With the addition of ssDNA, there is rapid dissociation of the CPC\textsubscript{W} complexes, but there was observable binding to the +18 to -55 region. In the presence of δ, the CPC\textsubscript{W} complex was not observed, and rapid dissociation of other bound complexes was observed upon addition of the ssDNA competitor.

To quantitate these effects, densitometry was performed on the autoradiograms. Three series of bands were quantified for each lane analyzed. The series of bands below the protection region, labeled "N," were used to normalize the intensity of the DNase I profiles among the lanes (Figure 26). This normalization corrected for lane variations and permitted direct comparisons. The series of bands labeled "O" were protected from DNase I in open promoter complexes but not in closed promoter complexes. Loss of
**Figure 26:** Dissociation of RNA polymerase-Alu156 complexes in the presence or absence of the δ subunit as detected by DNase I footprinting analysis. DNA fragments were incubated in the absence (lane with a minus sign) or in the presence of 6 μg RNA polymerase for 10 minutes (lanes with a plus sign). The lanes marked "10 s", "20 s", and "30 s" in the presence or absence of δ subunit received single-stranded M13 DNA 10, 20, and 30 seconds prior to DNase I treatment, respectively. The series of bands labeled "N" were quantified and used to normalize the intensity of the DNase I profiles among the lanes. Bands marked "O" were protected from DNase I in open promoter complexes protected from DNase I attack. The protection region labeled "T" was protected from DNase I in all RNA polymerase-promoter complexes (closed and open complexes). This figure contains representative data from a single experiment. The experiment was repeated at least three times to insure reproducibility of the results.
protection of this area was used as an indicator of the loss of open promoter complexes. The region labeled "T" was protected from DNase I in both open and closed promoter complexes. This region was used as a measure of all complexes. Each series of bands was quantified, and the results presented as a ratio of O/N to represent the open promoter complexes and T/N to represent the total number of complexes observed (Figure 26). For both the total complexes and the open promoter complexes, the presence of δ increased the rate of dissociation significantly. For the total complexes (Figure 27A) in the presence of δ, the fraction of bound complexes measured was initially comparable to those observed in the absence of δ. However, upon addition of the ssDNA competitor, there was a rapid dissociation of complexes in the presence of δ. These unstable complexes represent closed promoter complexes. As expected, this initial rapid dissociation was not observed when the open promoter complexes were quantified (Figure 27B). These data clearly demonstrate that the stability of all promoter complexes measured is affected by the presence of δ.

**The δ subunit does not alter the effect of curved DNA on DNA transcription.** The effects of curved DNA on RNA polymerase binding and transcription were observed in the presence of δ subunit (*i.e.*, all gel retardation assays and transcription assays in this research were done in the presence of δ subunit unless otherwise stated.) To demonstrate this fact, the single-round, runoff transcription assay using the temperature shift variation to measure the levels of CPC5 was conducted in the presence of δ. When single-round transcription was measured at each of the temperatures tested, no transcription was observed at 0°C or 10°C for any of the promoters tested (Figure 28A). As observed previously with RNA polymerase without δ (Figure 23), the Alu156 promoter was most efficiently transcribed followed by the two promoters with (A+T)-rich DNA upstream of the -35 region, the AluUp and AluUnc promoters. The AluExt promoter, which lacks curved DNA and (A+T)-rich DNA, was very inefficiently transcribed. When reactions were shifted to 37°C after the addition of ssDNA and the
Figure 27: Dissociation rate of RNA polymerase-Alu156 complexes detected by the DNase I footprinting. RNA polymerase-DNA complexes formed with (■) or without (●) the δ subunit were determined 10, 30, 90, 270, and 810 seconds after adding 7.5 μg of the single-stranded M13 DNA. Total complexes (panel A) and open promoter complexes (panel B) of RNA polymerase bound to Alu156 promoter were quantified and plotted as the fraction DNA bound relative to the time following single-stranded DNA addition as described in the Materials and Methods section. This figure contains representative data from a single experiment. The experiment was repeated at least three times to insure reproducibility of the results.
Figure 28: Temperature-shift transcription assay to detect stable closed complexes in the presence of the δ subunit. Transcribable promoter complexes were measured in a single-round, runoff transcription assay as a function of temperature. Representative data for the relative number of transcripts for Alu156 (●), AluExt (■), AluUnc (▼), and AluUp (▲) were determined by densitometry of an autoradiograph of a polyacrylamide gel containing [32P]-end labeled transcripts. The assay was conducted without (panel A) or with (panel B) shifting the reactions to 37°C to permit stable closed complexes to form open promoter complexes and initiate transcription. This figure contains representative data from a single experiment. The experiment was repeated at least three times to insure the reproducibility of the results.

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nucleotide mix, significant transcription was observed for the Alu156 promoter at 0°C and 10°C (Figure 28B). This transcription defines a closed promoter complex that was stable to ssDNA challenge, the CPC₅. The AluUp promoter had elevated levels of CPC₅ relative to AluExt confirming the positive contribution of (A+T)-rich DNA sequences to the binding of RNA polymerase. Although the δ subunit reduces the stability of RNA polymerase complexes, δ does not alter the ability of curved DNA to enhance transcription initiation. This enhancement appears to relate to ease in forming CPC₅ in the presence of δ with promoters with curved DNA.

If the role of the δ subunit is to reduce the interactions between RNA polymerase and the DNA, how does it benefit the cell in the process of transcription? It has been proposed that the δ subunit permits the RNA polymerase to stay free of unproductive complexes and to efficiently reach the open promoter complexes, which are able to initiate transcription (Achberger and Whiteley, 1981; Achberger et al., 1982; Juang and Helmann, 1994; 1995). This was recently demonstrated by comparing the effect of the δ subunit on a single-round transcription assay with a multiple-round transcription assay (Juang and Helmann, 1994; 1995). To confirm this finding with our Alu156 series of promoters, we compared runoff transcription in single-round and multiple-round formats (Figure 29). In the single-round, runoff transcription assay (Figure 29A), only open promoter complexes present when the single-stranded competitor DNA and nucleoside triphosphates were added would initiate and form a transcript. Since the δ subunit reduces the number of complexes present, transcription in this assay was reduced in the presence of δ. This effect was independent of the presence of curved DNA or (A+T)-rich DNA sequences upstream of the promoter. When tested in the multiple-round transcription assay, the presence of δ subunit stimulated transcription significantly (Figure 29B). This stimulation by δ was most likely due to efficient recycling of the RNA polymerase through all the intermediates between cycles of transcription (Juang and Helmann, 1994; 1995).
Figure 29: Comparison of single-round and multiple-round transcription assays in the presence or absence of the δ subunit. Transcripts formed in single-round (Panel A) and multiple-round (panel B) runoff transcription assays without (open bars) or with (filled bars) the δ subunit. Relative number of transcripts for Alu156, AluExt, AluUnc, and AluUp promoters were determined by densitometry of autoradiographs of a polyacrylamide gels containing [32P] end-labeled transcripts. Panel A and Panel B represent independent experiments conducted on the same day. This figure contains representative results from one of three repeats of this experiment.
DISCUSSION

*B. subtilis* RNA polymerase wraps the DNA upstream of the core promoter. The original Alu156 promoter contains A-tracts mediates curved DNA upstream of the -35 region. Previous binding studies have shown that the deletion of the curved DNA reduced the affinity of *B. subtilis* RNA polymerase for this promoter (McAllister and Achberger, 1988). In a separate study, binding of *B. subtilis* RNA polymerase to Alu156 was also affected by the orientation of the curved DNA relative to the -35 region. Effective RNA polymerase binding to a series of promoters in which DNA was inserted between the -35 region and the curved DNA was only observed for promoters that maintained the orientation observed for the Alu156 promoter (McAllister and Achberger, 1989). These data indicated that the curved DNA contributed to the formation of a specific RNA-polymerase-promoter complex. DNase I footprinting analyses were used to detect this complex. In addition to the typical region of protection, representing the tight binding of RNA polymerase to -10 and -35 regions, a distinct DNase I pattern was observed upstream. Beginning at the curved DNA and extending beyond -120, there were DNase I hypersensitive sites adjacent to sites protected from DNase I repeated mostly every ten base pairs in the DNA. This distinct pattern was attributed to DNA wrapped around RNA polymerase (Cheng, 1996). This pattern was observed for both the lower and the upper strands of DNA such that the DNase I hypersensitive sites fell on the same side of a cylindrical projection of the DNA.

Similar results were observed using the RNA polymerase from *E. coli* (Nickerson and Achberger, 1995; Jazbi, 1997). Wrapping of DNA around protein was first documented in eukaryotic systems (Shaw et al., 1976; Prunell et al., 1984; Luger et al., 1997).

The DNase I pattern attributed to DNA wrapping was discovered for DNA wrapped around the histone complexes. In these complexes, 146 base pairs are wrapped around the histone octamer in 1.65 turns of left handed superhelix axis. The statistical
preference observed for the DNA minor groove to face the histone octamer at (A+T)-
rich sequences indicates that certain sequences will impart a rotational orientation to the
DNA double helix when bound in a nucleosome (Luger et al., 1997).

In the present study, a refinement of the DNase I experiments is described. Previously, RNA polymerase binding conditions were optimized to document the "wrapped" RNA polymerase-promoter complex (Cheng, 1996). The present study examined RNA polymerase-promoter complexes under conditions that optimized RNA synthesis. The DNase I pattern observed closely resembled previous work except that the region from +1 to +18 of the lower strand of the promoter was protected from DNase I in the present study. DNase I footprints that extend to +18 are indicative of open promoter complexes, while the DNase I footprints for closed promoter complexes end around the +1 transcription initiation region. The presence of transcribable open promoter complexes as well as the wrapped promoter complex was confirmed based on potassium permanganate sensitivity (data not shown) (Spassky and Sigma, 1985; Spassky, 1992).

Model for DNA wrapping by RNA polymerase. Previous studies with B. subtilis RNA polymerase have shown that binding and open complex formation are enhanced by curved DNA associated with promoters (McAllister and Achberger, 1988, Stemke, 1993). Based on these and other studies in our laboratory, a model was proposed for the role of curved DNA in transcription initiation. It has been proposed that the series of A-tracks upstream the -35 region has dual functions. The runs would be predicted to be α subunit binding sites that allows the curved DNA to be in close proximity to the RNA polymerase. The A-tract mediated curvature would deflect the DNA helix, thus directing the upstream DNA around the enzyme and facilitate additional contacts as the DNA is wrapped around the RNA polymerase. With the DNA tightly wrapped around the RNA polymerase, the enzyme-promoter conformational changes permit untwisting of the DNA helix, a mandatory step before the separation of the strands at the -10 region.
Thus, curved DNA helps in DNA structuring via DNA wrapping leading to the formation of open complex for transcription initiation. It should be emphasized that this model predicts that DNA wrapping occurs with all promoters. However, only in the presence of curved DNA, are these complexes stable enough to footprint.

Curved DNA sequence appears to behave in a manner similar to that observed with upstream activators, such as CAP, which alter DNA conformation and allow upstream interactions. CAP and cAMP as a complex specifically binds sites positioned upstream of the -35 region of many promoters. Upon binding, CAP bends the DNA and interacts with the α subunits of the RNA polymerase. This association between CAP and the RNA polymerase wraps the DNA around the enzyme in a fashion analogous to what has been proposed for the curved DNA. Interestingly, the CAP binding sites for the lac and gal promoters, could be functionally replaced by intrinsically curved DNA (Bracco et al., 1989; Gratenberg and Crothers, 1991).

The present studies define the model in terms of the kinetic intermediates leading to transcription initiation. Kinetic studies have indicated the presence of at least two RNA polymerase-promoter complexes prior to open complex formation (RP₀) (Rosenberg et al., 1982; Roe et al., 1984; Roe, 1985; Buc and McClure, 1985; Duval-Valentin and Ehrlich, 1987).

\[
\text{R+P} \leftrightarrow \text{RP}_C^1 \leftrightarrow \text{RP}_C^2 \leftrightarrow \text{RP}_O
\]

The initial closed complex (RP_C¹) (Kadesch et al., 1982; Rosenberg et al., 1982), followed by an a second closed promoter complex (RP_C²) (Roe et al., 1984; Buc and McClure, 1985), in which the strands of the DNA are partially unwound but remain base paired. The latter complex is stable at room temperature (i.e., the most common complex formed below 20°C). RP_C² is not readily displaced by heparin or competing DNA templates (Buc and McClare, 1985; Spassky and Sigma, 1985; Schicker et al., 1990). The transition from RP_C² to RP_O becomes the rate limiting step below 20°C (Buc and McClure, 1985, Spassky and Sigma, 1985).
The present study has identified two distinct closed promoter complexes. Both complexes were observed at 0°C, a temperature at which open promoter complexes and transcription were not detectable. The closed promoter complex in which the DNA is wrapped around the RNA polymerase, CPC\textsubscript{W}, was observed to quickly dissociate in the presence of ssDNA. CPC\textsubscript{W} possesses all the characteristics described for RP\textsubscript{C1}, described above. This complex was shown to be stabilized in the presence of curved DNA upstream of the promoter (i.e., DNase I patterns indicative of DNA wrapping were difficult to identify in promoters lacking curved DNA).

The second closed promoter complex, CPC\textsubscript{S}, was relatively stable in the presence of ssDNA competitors. CPC\textsubscript{S} has the general characteristics ascribed to RP\textsubscript{C2}. It follows that for the Alu156 promoter, CPC\textsubscript{S} represents RP\textsubscript{C2} and CPC\textsubscript{W} represents RP\textsubscript{C1}, and that CPC\textsubscript{W} isomerizes into CPC\textsubscript{S} just as RP\textsubscript{C1} isomerizes into RP\textsubscript{C2}. Interestingly, there was no evidence of DNA wrapping in CPC\textsubscript{S} (Figure 22) suggesting that the DNA wrapping observed with CPC\textsubscript{W} contributed to the transition to CPC\textsubscript{S}. If DNA wrapping in CPC\textsubscript{W} (RP\textsubscript{C1}) contributes to the formation of CPC\textsubscript{S} (RP\textsubscript{C2}), one should be able to measure elevated production of CPC\textsubscript{S} for promoters with curved DNA. Since RP\textsubscript{C2} is the predominant stable complex below 20°C, an assay was devised to quantify this complex. A single-round transcription assay was used to limit transcription to RNA polymerase-promoter complexes formed at low temperature and to prevent new initiation by free RNA polymerase (Figure 23, panel A). The addition of ssDNA eliminated RP\textsubscript{C1} as it quickly dissociated. A temperature shift to 37°C was used to permit RP\textsubscript{C2} formed at low temperature to isomerize to open promoter complexes (RP\textsubscript{O}) and initiate RNA synthesis. Alu156, which possessed curved DNA, formed significantly greater numbers of CPC\textsubscript{S} (RP\textsubscript{C2}) relative to Alu156-derived promoters lacking curved DNA (Figure 23B). This suggests that the conformation of the wrapped RNA polymerase-promoter complexes, CPC\textsubscript{W} (RP\textsubscript{C1}), favors the transition to CPC\textsubscript{S} (RP\textsubscript{C2}), which in turn isomerizes to
RP₀. Thus, the curved DNA facilitated the formation of CPC₇ (RP₁₁) and indirectly the formation of CPC₅ (RP₆₂), two of the common rate limiting steps in transcription initiation.

Nature of the curved DNA and its interaction with RNA polymerase. The region of curved DNA in the Alu156 promoter contributes greatly to transcription initiation. The curved DNA region could influence RNA polymerase binding through the actual DNA curvature, the (A+T)-rich nature of the A-tract mediated curved DNA acting as an α subunit binding site, and/or the presence of a previously not described binding site for RNA polymerase. To address the nature of the curved DNA region of Alu156, the native curved DNA was replaced with a synthetic curved DNA sequence. While the synthetic DNA was designed with A-tracts to bend the DNA, no attempt was made to conserve the overall (A+T) richness observed in the native sequence (Figure 5). Since a single 4-6 base pair A-tract immediately upstream of the -35 region is the most commonly documented (A+T)-rich region in promoters from Gram-positive bacteria (Graves and Rabinowitz, 1986), a single A-tract was placed at this position in Alu1A. Starting at this point on the promoter, additional A-tracts were placed in phase with the DNA helical repeat to form the Alu2A and Alu3A promoters (Figure 5). The three A-tracts of Alu3A possessed significant curvature relative to the AluExt promoter, which lacked the A-tracts. The binding of RNA polymerase to this collection of promoters was examined using gel retardation assay and DNase I footprinting experiments. Alu3A promoter was bound by RNA polymerase more effectively than promoters containing one or two adenine tracts. The DNase I footprinting pattern indicative of DNA wrapping was observed when the original curved DNA was replaced with the synthetic curved DNA in Alu3A promoter. However, DNA wrapping was more prominent with the original curved DNA of Alu156, and little or no evidence of wrapping was found for AluExt, Alu1A or Alu2A promoters. In this study, there was a direct correlation between RNA polymerase binding, evidence of DNA
wrapping and the presence of DNA curvature. With this set of promoters, we found evidence for cumulative effects of phased A-tract inserted upstream of the -35 promoter region. This was clearly demonstrated at the level of RNA polymerase binding and transcription. The general relationship in terms of transcription initiation was the following:

$$\text{AluExt} \leq \text{Alu1A} < \text{Alu2A} < \text{Alu3A} < \text{Alu156}$$

While Alu3A was the most efficient of the promoters possessing synthetic curved DNA, it routinely exhibited only 70-80% of the promoter activity of Alu156 depending on the assay used to evaluate promoter function.

One could argue that the increased promoter function observed for Alu3A was due to the position of the third A-tract (around -65) relative to the core promoter and not because of the number of A-tracks immediately upstream of the -35 region. Two points can be addressed. First, Graves and Rabinowitz (1986) have reported that, the most common position to find a single A-tract in a collection of promoters from Gram positive organisms is immediately upstream of the -35 at positions -41 to -45. A single A-tract centered at -42 is observed much more frequently than a single A-tract in the -65 region. Additionally, in *B. subtilis* promoters, it is more frequent to find a single A-tract centered at -42 than to find two or three runs of A-tracks in phase with the helical repeat of the DNA (Stemke, 1993). So it is most likely that an A-tract located around the -40 region has the most significant effect on promoter function. For this reason, an A-tract centered at -42 was used to anchor the addition of A-tracks. Second, Based on binding and transcription studies (Cheng, 1996; McAllister and Achberger, 1998) performed in Dr. Achberger's lab, insertions of 11 and 21 bps between the -35 region and the first A-tract retained much of the promoter function observed for the original Alu156 promoter. However, the insertion of 21 bps (i.e., the first of these A-tracts was centered at -63) displayed significantly lower binding and fewer transcripts when compared to the promoter with the insertion of 11 bps even though properly aligned with the promoter.
In each of these promoters (i.e., Alu156, Alu+11, and Alu+21), there is an A-tract at the -63 region; however, promoter function decreased as the distance between the promoter and the curved DNA increased. This proximity effect indicates that the optimal positioning of the curved DNA in Alu156 is with the first A-tract centered at -42. There is no special significance of an A-tract located at the -65 region beyond its contribution to DNA curvature.

Two hypotheses were proposed to explain why the curved DNA of Alu3A did not achieve the level of stimulation observed for the native curved DNA of Alu156. It is possible that there are sequences within the curved DNA sequence that contribute to promoter function. An alternate possibility is that the alignment of the curvature relative to the core promoter of Alu3A and the original Alu156 is not the same.

Alignment of the synthetic curved DNA in Alu3A promoter. A remarkable feature of promoter activation mediated by intrinsic curved DNA sequence is the dependence of activation on the orientation of curved DNA relative to the promoter (McAllister and Achberger, 1989). Experiments in which the distance between the curved DNA and the -35 region of Alu156 was changed by short oligonucleotide insertions of 6-29 base pairs showed that most efficient mutant promoters contained insertions of 11 or 21 base pairs (i.e., insertions maintained the original rotational orientation while linearly displacing the curved DNA). On the other hand, insertions of 15 or 25 base pairs (i.e., the curved DNA was rotated away from the original orientation) resulted in the least efficient promoters (McAllister and Achberger, 1989). These results suggested that proper rotational orientation is the most crucial factor for the stimulation of transcription by the upstream curved DNA. In the present work, the synthetic curved DNA of Alu3A less was less effective than the native curved DNA. Therefore, two variants of Alu3A were made in which the curved DNA was moved one base closer to, Alu3A(-1), or further away from, Alu3A(+1), the -35 region of the promoter. A change of one base pair rotates the curve by about 34° relative to the promoter. DNase I footprinting pattern
indicative of DNA wrapping was similar for Alu3A promoter and its derivatives relative to the original Alu156 (Figure 16). However, DNA wrapping was more prominent in Alu156. A region of enhanced cleavage was observed at -70 for Alu156 and Alu3A(-1). These hypersensitive sites were weaker for Alu3A and the weakest for Alu3A(+1) relative to the original Alu156. These data were in agreement with results of gel shift assays where Alu3A(-1) was superior to Alu3A(+1) in RNA polymerase binding (Figure 11). The deletion of 1 base pair in Alu3A(-1) resulted in less than a ten percent increase in promoter binding relative to Alu3A, and binding was less than that observed for Alu156. This suggests that the orientation of the synthetic curved DNA in Alu3A relative to the core promoter was not far from optimum. Since the B. subtilis RNA polymerase exhibited a highest affinity to Alu3A(-1) relative to Alu3A, it would be interesting to construct a promoter with a deletion of two base pairs between the -35 region and the curved DNA and to test if Alu3A(-1) represented the optimal positioning of the curved DNA. As documented for the Alu156 promoter (McAllister and Achberger, 1989; Cheng, 1996), using a series of double-stranded oligonucleotide insertions between the -35 region and the curved DNA, there was little effect on promoter function if short nucleotide insertions maintained the curved DNA at or near the optimum orientation. Based on this previous work and the results obtained from gel retardation and DNase I footprinting analyses, it appears that the Alu3A and Alu3A(-1) are close to the optimal orientation.

Based on gel retardation studies, DNase I footprinting experiments, synthetic curved DNA sequences immediately upstream of the -35 region did not fully substitute for the native curved DNA. The second possibility that the (A+T)-richness of the native curved DNA region functions as a α subunit binding site was investigated. Based on our data, we support the idea that there are two elements of the DNA upstream of the -35 region of Alu156 that contribute to enhanced transcription, (a) the (A+T)-rich sequence...
that would favor binding of the α subunit and (b) the DNA curvature that facilitates the wrapping of upstream DNA around the RNA polymerase.

**Effect of (A+T)-rich region on DNA wrapping and transcription initiation.** It was shown that the UP element of *E. coli rrnB* promoter increases transcription by interacting with the α subunit of RNA polymerase (Ross *et al.*, 1993). *E. coli* RNA polymerase containing α subunit with C-terminal truncations of 94 amino acids failed to protect the UP element from DNase I digestion. Similar UP elements have been described for the *B. subtilis* flagellin promoter (Fredrick *et al.*, 1995). It has been suggested that phased A-tracts immediately upstream of the -35 region are simply the α-binding sites. To test the contribution of (A+T)-rich DNA on the function of Alu156, two unique promoters were studied. The AluUp promoter contains the UP element from the *E. coli rrnB* promoter in place of the curved DNA. The AluUnc promoter, in which the curvature of the DNA upstream of Alu156 was disrupted by four single base substitutions, retains the (A+T)-richness and most of the sequence identity of Alu156. DNase I cleavage patterns attributed to DNA wrapping was most evident for Alu156 promoter. Results of gel retardation assay showed that promoters with non-curved (A+T)-rich DNA displayed significant decrease in the binding affinity of RNA polymerase relative to Alu156. These results were consistent with the data obtained from DNase I footprinting experiments in which little evidence of DNA wrapping was observed with promoters where the curved DNA was deleted (*i.e.*, AluExt) or exchanged for non-curved DNA rich in (A+T) (*i.e.*, AluUp).

Similar results were observed with *E. coli* RNA polymerase (Jazbi, 1997). A short region of protection from DNase I ranged from -45 to -65 on the promoter containing the UP element, AluUp. This is the same general area characterized by DNase I footprinting as the (A+T)-rich α subunit binding site on the *rrnB* promoter (Ross *et al.*, 1993). Fredrick and his coworkers (1995) have demonstrated that transcription from the *B. subtilis* flagellin promoter is stimulated 20-fold by an upstream...
(A+T)-rich region (upstream promoter UP element) both in vivo and in vitro. The UP element increases the affinity of RNA polymerase for the flagellin promoter and stimulates transcription when initiation is limited by the rate of RNA polymerase binding. Since the original Alu156 has (A+T)-rich region immediately upstream of the -35 region, it is likely that this region functions as an α binding site. The AluUnc promoter, which has the same upstream DNA as Alu156 except for the four base substitution within the A-tracts (Figure 18), retained 60-70% of the promoter function depending on the assay (Figure 19). The upstream DNA of AluUnc was functionally analogous to the UP element, which are known α subunit binding sites. This conclusion was further strengthened by the results from single-round, runoff transcription assay (Figure 23). The relative number of transcripts as a function of temperature was determined. The AluExt promoter, which lacks curved DNA and (A+T)-rich DNA, was the weakest promoter in all temperatures. This is consistent with the result from gel retardation assay where RNA polymerase exhibited a very low affinity for this promoter. AluUnc and AluUp promoters did not form as many transcripts as the original Alu156. They were significantly better than the AluExt promoter. This highlights the importance of curved DNA in transcription initiation. The data supports that hypothesis that there are two elements of the DNA upstream of the -35 region of Alu156 that contribute to enhanced transcription, (a) the (A+T)-rich sequence that would favor binding of the α subunit and (b) the DNA curvature that facilitates the wrapping of upstream DNA around the RNA polymerase.

RNA polymerase-promoter interactions in the presence of the δ subunit. With respect to the δ subunit of B. subtilis RNA polymerase, the novel finding was the failure to detect the RNA polymerase-promoter complex possessing DNA wrapping. The DNase I footprinting profile for RNA polymerase without δ (Eσ) bound to Alu156 demonstrated the pattern of DNase I hypersensitive sites and sites protected from DNase I cutting indicative of DNA wrapping. The addition of purified δ
subunit to this RNA polymerase eliminated the DNase I pattern indicating DNA wrapping and reduced RNA polymerase-promoter complexes overall (Figure 26). It was hypothesized based on previous work that the δ subunit increased the rate of dissociation of all complexes, including the wrapped complex, such that it was not stable enough to be detected during the 30 second incubation with DNase I. To test this idea, the stability of RNA polymerase-promoter complexes was determined with and without the δ subunit. The filter binding assay clearly demonstrated the reduced stability of complexes formed in the presence of the δ subunit (Figure 25).

In a unique application of the DNase I footprinting assay, the dissociation of complexes detected by this technique was measured (Figure 27). The addition of δ increased the dissociation rates of all complexes detected (Figures 25 and 27). The demonstration that δ reduced the stability of complexes in the DNase I footprinting assay directly supported the hypothesis that the wrapped DNA complex, CPCW (RPCC1) was too unstable in the presence of δ to be detected. With the enzyme containing δ (Eσδ), there is a rapid equilibrium between free RNA polymerase, closed complexes, and open complexes. This is consistent with the proposal, based on competition experiments, that the various intermediates in transcription initiation are in rapid equilibrium at many B. subtilis promoters (Wipple and Sonenshein, 1992). The data presented here agree with observations reported by Achberger et al. (1982) on the effect of δ on RNA polymerase binding to a collection of B. subtilis bacteriophage promoters. These researchers have shown that even at strong promoters, Eσδ binds less tightly to DNA than enzyme lacking δ (Eσ) as judged by a reduced extent of protection against DNase I digestion. Furthermore, similar results were observed by Juang and Helmann (1994) where RNA-promoter contacts at the ilv-leu (P_{ilv}) and trns (P_{trns}) promoters were reduced upon the addition of δ subunit. Thus, the δ subunit decreases the affinity of RNA polymerase to the Alu156 promoter indicating that Eσ undergoes a δ
mediated conformational change reducing the number of contacts with promoter DNA and decreasing the half-life of promoter DNA complexes.

**The effect of δ on the in vitro transcription of Alu156 promoter.** The effect of δ on the rate of RNA synthesis as a function of temperature was investigated using runoff transcription assays (Figure 29). Under our standard reaction conditions, δ stimulates the amount of RNA synthesized from the original Alu156 several folds in multiple cycle reactions perhaps possibly due to increased rate of RNA polymerase recycling through the kinetic intermediates (Figure 29B). Similar results were observed by Juang and Helmann (1994) where δ stimulated transcription from the *ilv-leu* operon which was attributed to an increased efficiency of RNA polymerase recycling. One model suggests that, in the absence of δ, product RNA appears to bind RNA polymerase and inhibit recycling. Product inhibition has been reported in various studies (Fox et al., 1985; Hsu et al., 1991; Krakow, 1966; Tissieres et al., 1963). The EσA binds to 3' end of RNA in the product binding site, thus stopping the production of any new RNA. The subunit δ acts to speed recycling by overcoming product inhibition of RNA synthesis and thereby stimulate transcription by displacing RNA from RNA polymerase-RNA binary complexes (Lopez de Saro and Helmann, 1995).

Alternatively, δ may increase the transition between kinetic intermediates by reducing the stability of any one complex. Biochemical studies of the effects of δ protein on in vitro transcription demonstrated a stimulation in transcription specificity which can be attributed to more selective interactions with strong promoter sites due to preferential destabilization of non-promoter and weak promoter complexes (Achberger and Whiteley, 1981; Achberger et al., 1982, Dobinson and Spiegelman, 1987). It has been suggested that δ, like *E. coli* σ70, can act to release non-specifically bound core enzyme from DNA (Chamberlin, 1982; Helmann and Chamberlin, 1988).

Although the δ subunit reduced the stability of all complexes such that the CPCw (RPc1) complex was not detectable by DNase I footprinting, the effect of
curved DNA on transcription initiation was observed. In fact, all gel retardation experiments and transcription assays described in this dissertation, unless otherwise stated, were conducted in the presence of the δ subunit.

The temperature-shift transcription assay was used to detect stable closed complexes in the presence of δ subunit. The original Alu156 and promoters lacking the curved DNA were used in this assay. Addition of δ causes a decrease in the relative number of transcripts produced by all promoters at all temperatures (Figure 23) relative to those observed when Es was used (Figure 28). In the temperature-shift reactions (Figure 28B), the original Alu156 was the only promoter to form significant number of transcripts at 0°C even in the presence of δ subunit, whereas (A+T)-rich promoters did not display the same levels of transcription at any tested temperature. Therefore, the curved DNA favors the formation of stable closed complexes that quickly isomerized into open complexes. Despite the inhibitory effect of δ on promoter binding as judged by filter binding assay and DNase I footprinting, it did not alter the complexes formed or the effect of curved DNA on transcription. The observations are consistent with a model in which the δ subunit participates together with the σ subunit to increase promoter selectivity by inhibiting binding and open complexes formation at all but the strongest promoters.

The findings with δ in no way alter the model for the role of DNA wrapping in transcription initiation. In this model, the RNA polymerase wraps the DNA around itself to form CPCw(RPc1) in a reaction facilitated by (a) binding of the α subunit to an (A+T)-rich site upstream of the promoter and (b) DNA curvature, which directs the DNA around the enzyme. CPCw (RPc1) isomerizes to CPCs (RPc2), a relatively stable closed promoter complex, which lacks DNA wrapping. CPCs (RPc2) isomerizes to form the open promoter complex, RP0, at which transcription initiation occurs. In the absence of (A+T)-rich DNA or DNA curvature, these reactions still occur albeit less efficiently.
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APPENDIX A

THE SUMMARY OF DENSITOMETRIC SCANS OF THE DNASE I FOOTPRINTING ANALYSIS OF ALU156 PROMOTER

Panels A and B represent the densitometric analyses of the footprint obtained for the lower (template) strand and the upper (non-template) strand DNA of Alu156 promoter. Lower strand and upper strand of the promoter are footprinted in the absence (traces with a solid black line) and presence (traces with dotted black line) of RNA polymerase. In both panels, the two densitometric scans have been superimposed to highlight regions of protection and enhanced cleavages. The scans are numbered in 10 base pair intervals to approximate the location of the DNA bands relative to the transcription start site (+1).
APPENDIX B

THE SUMMARY OF DENSITOMETRIC SCANS OF THE DNASE I FOOTPRINTING ANALYSIS OF ALUEXT PROMOTER

A.

B.

Panels A and B represent the densitometric analyses of the footprint obtained for the lower (template) strand and the upper (non-template) strand DNA of AluExt promoter. Lower strand and upper strand of the promoter are footprinted in the absence (traces with a solid black line) and presence (traces with dotted black line) of RNA polymerase. In both panels, the two densitometric scans have been superimposed to highlight regions of protection and enhanced cleavages. The scans are numbered in 10 base pair intervals to approximate the location of DNA bands relative to the transcription start site (+1).
APPENDIX C

DENSITOMETRY SCANS OF THE DNASE I FOOTPRINTING ANALYSIS OF THE UPPER STRAND OF ALU156 AND ALU3A PROMOTERS

Panels A and B represent the densitometric analyses of the DNase I digestion pattern obtained for Alu156 in the absence of (panel A) or presence (panel B) of 1.2 μg/μl RNA polymerase. Similarly, panels C and D represent the densitometric analysis of Alu3A promoter without (panel C) or with (panel D) of 1.2 μg/μl RNA polymerase. The scans are listed in 10 base pair intervals to approximate the location of DNA bands relative to the transcription start site (+1).
APPENDIX D

THE SUMMARY OF DENSITOMETRIC SCANS OF THE DNASE I FOOTPRINTING ANALYSES OF THE UPPER STRAND OF ALU156, ALUEXT, AND PROMOTERS WITH VARIANT NUMBERS OF SYNTHETIC CURVED DNA

Panels A, B, C, and D represent the densitometric analyses of the DNase I footprints obtained for the Alu156, AluExt, Alu1A, and Alu2A promoters, respectively. In all panels, the upper (non-template) strand of each promoter was footprinted in the absence (trace with a solid black line) or the presence (traces with dotted black line) of RNA polymerase. The two densitometric scans have been superimposed to highlight regions of protection and enhanced cleavages. The scans are numbered in 10 base pair intervals to approximate the location of DNA bands relative to the transcription start site (+1).
APPENDIX E

THE SUMMARY OF DENSITOMETRIC SCANS OF THE DNASE I FOOTPRINTING ANALYSES OF THE LOWER STRAND OF ALU156, ALUEXT, AND (A+T)-RICH PROMOTERS

Panels A, B, C, and D represent the densitometric analyses of the DNase I footprints obtained for the Alu156, AluExt, AluUnc, and AluUp promoters, respectively. In all panels, the lower (template) strand of each promoter was footprinted in the absence (trace with a solid black line) or the presence (traces with dotted black line) of RNA polymerase. The two densitometric scans have been superimposed to highlight regions of protection and enhanced cleavages. The scans are numbered in 10 base pair intervals to approximate the location of DNA bands relative to the transcription start site (+1).

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APPENDIX F

DENSITOMETRIC SCANS OF THE DNASE I FOOTPRINTING ANALYSIS OF ALU156 IN THE PRESENCE OF SINGLE-STRANDED M13 DNA AT 37°C

A.

B.

C.

D.

Panels A and B represent the densitometric analyses of the DNase I digestion pattern obtained for the Alu156 promoter in the absence (panel A) or presence (panel B) of 1.2 μg/μl RNA polymerase. Panels C and D represent reactions in which RNA polymerase-Alu156 complex was challenged with single-stranded M13 DNA for 10 and 30 seconds prior to DNase I digestion, respectively. The scans are listed in 10 base pair intervals to approximate the location of DNA bands relative to the transcription start site (+1).
APPENDIX G

DENSIOMETRIC SCANS OF THE DNASE I FOOTPRINTING ANALYSIS OF ALU156 IN THE PRESENCE OF SINGLE-STRANDED M13 DNA AT 0°C

A.

B.

C.

Panels A and B represent the densitometric analyses of the DNase I digestion pattern obtained for the Alu156 promoter in the absence (panel A) or presence (panel B) of 1.2 μg/μl RNA polymerase. Panels C and D represent reactions in which RNA polymerase-Alu156 complex was challenged with single-stranded M13 DNA for 30 seconds prior to DNase I digestion, respectively. The scans are listed in 10 base pair intervals to approximate the location of DNA bands relative to the transcription start site (+1).
APPENDIX H

DENSITOMETRIC SCANS OF THE DNASE I FOOTPRINTING ANALYSIS OF ALU156 WITH OR WITHOUT THE δ SUBUNIT

Panels A and B represent the densitometric analyses of the DNase I digestion pattern obtained for the Alu156 promoter in the absence (panel A) or presence (panel B) of 1.2 μg/μl RNA polymerase. Traces that represent reactions in which the δ subunit was added are labeled C and D. δ subunit was mixed with the core enzyme before incubation with Alu156 promoter. The concentration of the δ subunit was 0.03 μg/μl (panel C) and 0.06 μg/μl (panel D). The scans are listed in 10 base pair intervals to approximate the location of DNA bands relative to the transcription start site (+1).
VITA

Murad Mohammed Odeh was born on April 20, 1968 in Kuwait city, Kuwait. He lived in Kuwait until the age of 18. After that, he moved to Irbid, Jordan, to attend Al-Yarmouk University beginning in the Fall of 1986. Four years later, Murad received a bachelor of science degree in Biology. Shortly thereafter, in the Fall of 1990, Murad M. Odeh enrolled in the graduate school of the same University. He earned his Master of science degree in Molecular Biology in January, 1993. After his graduation, Murad took a position in the Department of Applied Biological Sciences at Jordan University of Science and Technology in Ramtha, Jordan, where he worked as a teaching and research assistant. In January of 1995, he enrolled in the Graduate School of Louisiana State University where he is currently a candidate for the degree of Doctor of Philosophy in Microbiology.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Murad M. Odeh
Major Field: Microbiology
Title of Dissertation: The Role of Curved DNA in Bacillus subtilis RNA Polymerase-promoter Interactions

Approved:

[Signatures]

Major Professor and Chairman
Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination: July 30, 1998