DNA Wrapping and the Nature of Interaction Between E. Coli RNA Polymerase and Promoter DNA.

Parisa Jazbi

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DNA WRAPPING AND THE NATURE OF INTERACTION
BETWEEN E. coli RNA POLYMERASE AND PROMOTOR DNA

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfilment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Microbiology

by
Parisa Jazbi
B.S., Shiraz University, 1991
December 1997
In the name of God most merciful most gracious

DEDICATION

To my parents
for their love and faith in me
ACKNOWLEDGMENTS

I am greatly indebted to my major adviser who has been a significant mentor. His constant support, guidance, and encouragement made this work possible.

I would also like to thank the members of my committee, Dr. Randall Gayda, Dr. Ronald Siebeling, Dr. Gregory Jarosik, Dr. Gregg Pettis, Dr. Ding Shih, and Dr. David Senior for their invaluable and sensible advice.

My thanks also to my fellow graduate students in the microbiology department for their friendship and encouragement.

My brother, Ali deserves a special thank you for being my inspiration and incentive to finish this work.

Most of all, I am grateful to my husband, without whose love and belief in me I never could have completed this degree. He believed in me when I had a hard time believing in myself.
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ABSTRACT

Regulation of transcription most often occurs at the stage of initiation. RNA polymerase binding to the transcription start site, the promoter, is influenced by many nucleotide sequence elements. The predominant recognition sequences are those bound by the σ subunit of RNA polymerase located at -10 and -35 relative to initiation site of most promoters. Another element involved in this regulation is intrinsic DNA curvature. This study examined the contribution of intrinsically curved DNA immediately upstream of the promoter to the interaction between *Escherichia coli* RNA polymerase and this DNA. DNase I footprinting analysis confirmed that RNA polymerase wraps DNA upstream of the promoter around the enzyme. The nature of interaction between DNA upstream of promoter and RNA polymerase was explored using addition of NaCl. The wrapped complex was not observed at NaCl concentration above 150 mM suggesting the electrostatic, sequence-independent nature of the interaction. Study of the effect of temperature on DNA wrapping and open promoter complex formation demonstrated the existence of closed, wrapped complexes. No wrapped complexes survived a 30 second heparin challenge indicating the absence of wrapped open complexes. The above data provided evidence that DNA wrapping occurs prior to open complex formation. Promoters containing an AT-rich region or the UP element of ribosomal RNA promoter *rrnBP1* were constructed. Using a gel retardation assay, the relative affinity of RNA polymerase for these promoters was compared to that observed for curved DNA-containing promoter. The promoter containing curved DNA displayed the highest binding to RNA polymerase. The
presence of curved DNA favored the formation of the wrapped complex. A run-off transcription assay limited to a single round of initiation examined the efficiency of transcription for these promoters as a function of temperature. Relative to promoters lacking curved DNA, the promoter with curved DNA formed significantly more heparin-resistant, closed complexes at low temperature. These complexes could quickly isomerize to open complex at 37°C. We propose that curved DNA facilitates wrapping of DNA around RNA polymerase and enhances the transition from a heparin sensitive closed complex to a heparin resistance closed promoter complex.
INTRODUCTION

The regulation of gene expression is essential to the efficiency and economy of bacterial growth. Control of the activity of bacterial genes usually starts at the stage of transcription. The controlled step in this process is commonly the initiation of transcription. The least amount of energy and other resources are used by controlling the very first step of transcription.

Several elements are involved in the multiple step process of initiation of transcription. These components are common among several species of bacteria, including *Escherichia coli* and *Bacillus subtilis*. DNA dependent RNA polymerase binds to the promoter, a specific sequence of DNA, with or without the regulatory proteins that repress or activate transcription. Most of the interactions between the RNA polymerase and the promoter DNA are mediated through consensus DNA sequence elements, such as those commonly found at -10 and -35 relative to the site of transcription initiation. These interactions are involved in the separation of the DNA strands. Other DNA elements, such as intrinsically curved DNA, have been shown to affect the initiation of transcription. Involvement of these elements in regulation of transcription initiation can be further studied using *in vitro* systems.

**DNA-dependent RNA polymerase.** RNA polymerase is the cell's central processing unit. This enzyme was discovered in 1959 by Weiss and Gladstone. RNA polymerase copies the information from a DNA template to RNA molecule specifically. It synthesizes an RNA molecule that is complementary to the DNA template. The RNA polymerase of *E. coli* is composed of a core enzyme with the subunit structure of α2ββ' (Chamerlin, 1982; McClure,
1985) and one of the several species of $\sigma$ subunit which are involved in the specific promoter recognition (Helmann and Chamberlin, 1988). The catalytic site of RNA polymerase is located on the $\beta$ (150,619 Daltons) subunit (Glass et al., 1982), while RNA polymerase binds to the DNA nonspecifically via the $\beta'$ (155,162 Daltons) subunit (Fukuda and Ishihama, 1979).

Alpha ($\alpha$) subunits are involved in assembly of the RNA polymerase (Ishihama, 1981), and also in protein protein interactions with positive regulators (Ishihama, 1992). E. coli RNA polymerase with deletion in the C-terminal one third of the $\alpha$ subunit is enzymatically active, however, some activator dependent promoters cannot be transcribed by this mutant RNA polymerase. This suggests that the C-terminal region of $\alpha$ interacts with some transcription factors (Igarashi and Ishihama, 1991). The cyclic AMP receptor protein (CRP) contacts the $\alpha$ subunit of RNA polymerase when activating transcription at "class I promoters" in which the CRP binding site is located upstream of the -35 region. The targets for CRP interaction also reside in the C-terminal domain (CTD) of the $\alpha$ subunit (Chen et al. 1994; Zhou, et al., 1994; Zou et al., 1992). In addition, for a number of other bacterial activators, such as AraC, OxyR, PhoB, FNR, and integration host factor (IHF), it has been shown that they mediate their effect via the $\alpha$ CTD (Ishihama, 1992). The function of the C-terminal domain of the $\alpha$ subunit of E. coli RNA polymerase in basal expression and integration host factor-mediated activation of the early promoter of bacteriophage MU has been recently studied by Vanulsen et al.(1997). The results of this study indicates that interaction of the $\alpha$ CTD with DNA is involved not only in the IHF mediated activation of
early promoter but also in maintaining the basal level of transcription from this promoter. It was shown in $rnmB$ P1 promoter that sequences between -40 and -60, the so called "UP element", increases transcription by interacting with the $\alpha$ subunit of RNA polymerase (Ross et al., 1993; Gaal et al., 1996). RNA polymerase lacking the C-terminal domain of the $\alpha$ subunit was unable to contact the UP element, and therefore, transcription from $rnmB$ P1 promoter was less efficiently initiated (Ross et al., 1993). In the same study, it was shown that purified $\alpha$ binds specifically to the UP element. The binding was diminished when mutated $\alpha$ was used. Therefore, it can be concluded that the UP element represents a third promoter recognition region and that $\alpha$ acts directly in promoter binding.

Six different $\sigma$ subunits have been found in association with $E. coli$ polymerase. All these sigma factors play an important role in the specificity of the transcription initiation. Alternative $\sigma$ subunits control the transcription of coordinately regulated sets of genes distinct from those recognized by the primary $\sigma$ factor (Doi and Wang, 1986; Losick et al. 1986). In exponentially growing cells, most genes are transcribed by $\sigma^{70}$ (70,263 Daltons). Genes for heat shock proteins are transcribed by $\sigma^{32}$ (Grossman et al., 1984; Cowing et al., 1985; Fujita et al., 1987). A second heat shock sigma factor, $\sigma^{24}$, has been identified as a requirement for survival of high temperatures. The $\sigma^{24}$ is required for transcription of the genes which are controlled by the availability of nitrogen source (Garcia et al., 1977). The promoters of the genes that are expressed only in stationary growth phase can be recognized by $\sigma^{88}$ (Tanaka et al., 1993; Heggearonis, 1993). Genes for flagellar synthesis and chemotaxis are controlled by $\sigma^{28}$ (Arnosti et al., 1989). Recently, evidence for contact
between CRP and $\sigma^{70}$ subunit of E. coli RNA polymerase has been published for class II promoters (Ruzhonskl et al., 1995). The data indicates the role of the $\sigma^{70}$ subunit in transcription activation.

Unlike E. coli, B. subtilis undergoes a unique developmental process, called sporulation. This process is directed by a cascade of sigmas which lead to the temporal activation of different sets of genes during sporulation (Losick and Pero, 1981; Doi, 1982). B. subtilis RNA polymerase is very similar in core subunit composition to that of E. coli. However, B. subtilis also contain an additional polypeptide, the $\delta$ subunit (20,400 Dalton) (Doi, 1982; Lampe et al., 1988) and two $\omega$ subunits. The $\delta$ subunit is responsible in promoter discrimination. It appears that the $\delta$ subunit allows RNA polymerase to differentiate between strong and weak promoters (Achberger and Whiteley, 1982; Whiteley et al., 1982). The $\sigma$ subunit may be required for rapid recycling of the core RNA polymerase after termination (Juang and Helmann, 1994). The majority of cellular transcription is controlled by $\sigma^A$, the major vegetative sigma of B. subtilis (Losick et al., 1986). The $\sigma^D$ is involved in flagellar synthesis, chemotaxis, and nutrient stress responses (Helmann and Chamberlin, 1987; Helmann et al., 1988). In addition to $\sigma^A$, $\sigma^B$, $\sigma^C$ (Wiggs et al., 1981) and $\sigma^D$ (Haldemwang and Losick, 1980) are associated with vegetatively growing cells. Recently, three minor sigmas, $\sigma^X$, $\sigma^Y$, and $\sigma^W$, involved in the control of gene expression in response to environmental stresses were discovered (Huang et al., 1997). There are other sigmas associated with sporulating B. subtilis, including $\sigma^H$, $\sigma^F$, $\sigma^E$, $\sigma^G$, and $\sigma^K$ factors (Losick and Stragier, 1992). During sporulation the cell undergoes an asymmetric septation
that gives rise to a small cell destined to become the endospore and a larger, terminally differentiated mother cell. The $\sigma^H$ is a minor sigma factor involved in the transcription of both vegetative and sporulating genes. In this process, the inactive form of $\sigma^F$ is transcribed by $\sigma^H$ in the mother cell before the asymmetric cell division. Activation of $\sigma^F$ after septation directs the expression of $\sigma^G$. The $\sigma^G$ is produced in forespore and involved in transcription of forespore specific genes. The active sigma in mother cell, $\sigma^E$, is required for prespore formation. Endospore coat proteins are the product of cot genes. The $\sigma^E$ is necessary for expression of these genes in the mother cell.

Alternative sigma factors also have been found in phage SP82 or SPO1 infected B. subtilis cells. During the infection process, phage gene expression is controlled by a cascade of sigmas. The host $\sigma^A$ RNA polymerase is required for expression of early phage genes including gp28, which encodes $\sigma^{628}$. The $\sigma^{628}$ displaces $\sigma^A$ on the RNA polymerase shutting down host and early phage gene expression. The middle phage gene promoters are transcribed by $\sigma^{628}$ RNA polymerase. Among the middle genes are the gp33 and gp34, the gene products of which form a sigma, $\sigma^{33,34}$, these new sigmas displace $\sigma^{628}$ from the RNA polymerase shutting down middle phage gene expression and permitting late phage gene expression. Thus, the temporal gene expression during phage development is regulated by alternate $\sigma$ factors (Duffy et al., 1975; Talkington and Pero, 1978; Lee and Pero, 1981; Geidushik and Ito, 1982).

Promoters. Promoters are specific sequences of DNA located upstream of transcription start sites. RNA polymerase recognizes and binds to the promoter and initiates
transcription. The level of expression of a gene is greatly affected by the sequence of the promoter (Galas et al. 1985). The sequence comparison of many promoters has generated consensus sequence for particular RNA polymerase. The consensus sequence for *E. coli* σ^70^ holoenzyme is TATAAT (the -10 region) located about 10 base pairs upstream of the transcription start site and TTGACA (the -35 region) located 17 base pairs upstream of the -10 region (Rosenberg and Court, 1979; Siebenlist et al. 1980; Hawley and McClure, 1983; Harley and Reynolds, 1987). The role of -10 and -35 regions in the initiation of transcription has been studied. The -10 region is involved in DNA melting, while the -35 region plays a role in initial binding of RNA polymerase. In addition to binding affinity, the rate of open promoter complex formation is also affected by base substitution in -35 region (Hawley and McClure, 1982; Kobayashi et al., 1990). Genetic studies have shown that two regions of most σ factors are involved in interaction with the -10 and -35 regions of promoters (Waldburger et al., 1990; Siegle, et al., 1989; Kenney and Moran, 1991). In fact, amino acid substitution in the conserved sequences of σ which interact with the -35 region can alter promoter specificity. Thus, holoenzyme containing such a mutant σ recognizes promoters which normally are not transcribed by wild-type holoenzyme (Schmidt et al., 1990). It has been also demonstrated that holoenzyme containing alternate σ subunits recognizes unique promoters with different consensus sequences (Doi and Wang, 1986).

Several elements are responsible for promoter strength or the relative transcriptional effectiveness. Promoter strength is profoundly affected by mutation in the -10 and -35 region. Mutations causing divergence from the consensus sequence reduce the promoter strength, while mutations increasing the level of homology exhibit enhanced
promoter activity (McClure, 1985; Hawley and McClure, 1983; Harley and Reynold, 1987). The actual sequence of spacer DNA does not play a major role in promoter strength, however the distance between two consensus regions is critical for productive interaction between RNA polymerase and promoter.

Investigators have identified an additional conserved promoter sequence immediately upstream of the -10 region (Moran et al., 1982). A sequence around the +1 promoter region has been also identified to affect transcription in B. subtilis but not in E. coli (Henkin and Sonenshein, 1987).

Recently it has been established that there is a third important sequence element, in addition to the -35 and -10 elements, at some E. coli promoters (Ross et al., 1993). The exceptional strength of these promoters (ribosomal RNA genes) is due to the UP element, an AT-rich sequence of 20 base pairs located immediately upstream of the -35 region or the UP element. Fredrick et al. (1995) also demonstrated that transcription from the B. subtilis flagellin promoter is stimulated by an UP element both in vivo and in vitro. It is likely that promoter strength is a function of all promoter elements, with very strong promoters having near consensus elements while weaker promoters deviate significantly from the consensus.

Although the major forms of the RNA polymerase from E. coli and B. subtilis recognize the same consensus sequences (Moran et al., 1982; Galas et al., 1985; Graves and Rabinowitz, 1986), some differences have been reported for B. subtilis RNA polymerase. B. subtilis RNA polymerase generally requires DNA sequences that are more similar to the consensus sequence. In addition, inefficient utilization of E. coli promoters has been observed when they are introduced to B. subtilis (Wigg et al., 1979; Lee et al., 1980; Henkin
and Sonenshein, 1987; Whipple and Sonenshein, 1992). In contrast, \textit{B. subtilis} promoter can be utilized efficiently in \textit{E. coli}. Investigators have identified an AT-rich sequence upstream of the -35 region in many \textit{B. subtilis} promoters, including promoters for early gene expression in phage SPO1, which may be responsible for functional differences between \textit{E. coli} and \textit{B. subtilis} (Moran et al., 1982). To be fully functional, many promoters need additional sequences where regulatory proteins bind. These proteins can act as repressors or activators and regulate promoter function (Pabo and Sauer, 1984).

**Transcription initiation kinetics.** \textit{E. coli} is the traditional system for the study of transcription initiation kinetics. Transcription initiation by RNA polymerase is a complicated process comprised of a series of defined biochemical intermediates (Chamberlin, 1974; VonHipple et al., 1984; McClure, 1985). Two intermediates were involved in the original model. In this model, after binding of RNA polymerase (R) to the promoter (P), first a closed promoter complex is formed (RP\textsubscript{c}). The close complex then is isomerized to open promoter complex (RP\textsubscript{o}) in which the DNA strands of the promoter region are melted (Sienbenlist, 1979; Kirkegaard et al., 1983), exposing the template strand to RNA polymerase for RNA synthesis.

\[
\text{R + P } \xrightarrow{\text{RP}_c} \xrightarrow{\text{RP}_o} \text{RNA synthesis}
\]

The existence of at least two intermediate complexes before open promoter complex was documented (Rosenberg et al., 1982; Roe et al., 1984; Roe, 1985; Buc and McClure, 1985; Duval-valentin and Ehrlich, 1987). In most recent kinetic scheme RNA polymerase first binds to promoter and forms a closed complex. A conformational change, possibly

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leading to partial unwinding of the helix, forms a second closed complex, \( PR_{c2} \). Although closed promoter complexes are normally sensitive to heparin, this complex is heparin resistant. Heparin is a polyanion with high affinity for RNA polymerase (Walter et al., 1967). Isomerization later on leads to open promoter complex formation in a temperature-dependent process. Increasing the temperature activates the isomerization and drives the open promoter complex formation. In \( E. coli \) abortive RNA synthesis has been identified as an intermediate step between open promoter complex formation and productive RNA synthesis (Carpousis and Gralla, 1980). RNA polymerase of \( E. coli \) can go through multiple cycles of abortive initiation. After each cycle, a short RNA oligomer is released from the transcription complex. Usually after synthesis of 9 to 11 bases of RNA, promoter clearance occurs and the RNA polymerase complex enters the productive state (Grachev and Zaychikov, 1980; Streney and Crother, 1985; Garpouisis and Gralla, 1985). After the RNA polymerase clears the promoter, the \( \sigma \) subunit is released, and the elongation phase begins. The process ends with RNA chain termination.

There are many useful techniques to study thermodynamics and kinetics of specific RNA polymerase-promoter interactions. Binding assays, run-off transcription (Strauss et al., 1980; Rose et al., 1984) and abortive initiation (Hawley and McClure, 1982) are among the widely used techniques in this field. Chemical probes have been very useful to investigate RNA polymerase-DNA complexes. DNA melting has been studied using potassium permanganate which is known to react prefentially with pyrimidines in single-stranded DNA. This chemical probe can be used to detect DNA melting and open promoter complex formation both \textit{in vitro} and \textit{in vivo} (Sasse-Dwight and Gralla, 1989; Kainz and
Information about DNA conformation and accessibility to solvent and the presence of single-stranded DNA in open promoter complex has been accumulated using 1,10-phenanthroline-copper. There are some other chemical probes, such as hydroxy radical which has been used in the investigation. DNase I footprinting analysis has helped to demonstrate the interaction between RNA polymerase and promoter region and to define the kinetic intermediates of transcription initiation. DNase I cleavage patterns are different for open and closed complexes. Gel retardation analysis (Crothers, 1987; Gamer and Revzin, 1986) also known as gel shift assay can be used to determine the binding affinity of RNA polymerase for a promoter.

**Regulators of transcription.** Regulation of transcription initiation can be influenced by components in addition to the -10 and -35 regions. These components include proteins or DNA structural factors which either increase or decrease promoter activity and the initiation of transcription. *E. coli* CRP is a structurally characterized transcription activator protein (Kolb et al., 1993). In the presence of the allosteric effector cyclic AMP, CRP binds to specific DNA sites located near or in CRP-dependent promoters. Indeed, the binding of RNA polymerase to the promoter is enhanced by contact with bound CRP. CRP also stimulates transcription by bending the DNA. The bend induced by CRP, is estimated to be about 100° to 130° (Tompson and Landy, 1988; Zinkle and Crothers, 1990). The binding of CRP and of RNA polymerase are cooperative because they bend DNA in the same direction. There are two classes of CRP-dependent promoters. In class I, the DNA site for CRP is located upstream of the DNA site for RNA polymerase. The best characterized class I promoter is the *lacP1* promoter. In class II, however, the binding site
for CRP overlaps the DNA site for RNA polymerase. The galP1 promoter is an example of class II. Amino acids 156-164 of CRP constitute an activator region essential for transcription activation at both class I and class II CRP-dependent promoters, but it is not essential for DNA binding and DNA bending (Bell et al., 1990; Eschenlauer and Reznikoff, 1991; Zhou et al., 1993; Niu et al., 1994; Niu et al., 1996). For both class I and class II promoters, the activating region functions through protein-protein interaction with RNA polymerase. It is now believed that CRP interacts with the RNA polymerase α subunit C-terminal domain (αCTD) and facilitates the binding of αCTD to DNA adjacent to CRP (Igarashi and Ishihama, 1991; Kolb et al., 1993; Chen et al., 1994; Belyaeva et al., 1996). For class I promoters, the interaction between the activating region and αCTD appears to be the entire basis of transcription activation. The CRP-induced bend in class I tends to become localized at an apical loop of supercoiled DNA, thus helping the DNA to wrap around the promoter-bound RNA polymerase. In class II, the transcription activation requires not only the interaction between the activating region and αCTD, but also an interaction between a second site in the activating region of CRP and the RNA polymerase σ subunit N-terminal domain (Niu et al., 1996). This new finding establishes that an activator can interact with multiple targets within the transcription machinery and thereby affect multiple steps of transcription initiation.

Another example of a regulatory protein which can bend the DNA is the P4 protein of B. subtilis phage φ29. This protein is responsible for the switch from early to late transcription in the bacteriophage development. Protein P4 is produced at an early stage of infection and activates the transcription from the promoter for late genes called PA3.
This promoter is inactivated in the absence of protein P4. Activation of transcription in the presence of P4 is via stabilizing the binding of \textit{B. subtilis} RNA polymerase to the late promoter as a closed complex (Nuez et al., 1992). There is also evidence that P4 protein interacts directly with RNA polymerase at the PA3 promoter (Nuez et al., 1991; 1992; Serrano et al., 1991).

It has been shown recently that N4ssB, a single stranded DNA binding protein encoded by bacteriophage N4, interacts with the carboxyl terminus of the RNA polymerase \(\beta'\) subunit (Miller et al., 1997). N4ssB activates transcription by the \textit{E. coli} \(\sigma^{70}\) RNA polymerase at the N4 late promoter.

The level of transcription activation is also influenced by DNA supercoiling. It has been observed that supercoiling may either increase or decrease transcription activation in some genes. Nevertheless, many other genes are not influenced by these phenomena (Pruss and Drelica, 1989). DNA supercoiling may facilitate binding of RNA polymerase to the promoter and activator protein to the DNA. Transcription initiation at the lacP1 promoter of \textit{E. coli} is assisted by DNA supercoiling in a CRP-dependent fashion (Meiklejohn and Gralla, 1989).

Negative regulation of transcription can be also mediated through regulatory proteins. In 1961 Jacob and Monad proposed that a regulator gene produces a repressor that can interact with a DNA segment called the operator. Upon this interaction, initiation of transcription is prevented. This type of negative control has been observed for the lac operon. The product of the \textit{lac I} gene is an allosteric repressor protein with two binding sites. One binding site is for the operator region and the other is for the inducer molecule...
(allo-lactose). Upon binding of the *lac* repressor to the operator region, RNA polymerase is prevented from initiating transcription. However, in the presence of an inducer molecule, this molecule binds to the repressor and alters the structure of the repressor so it no longer binds to the operator. Thus the operator is unoccupied and the RNA polymerase can bind the promoter and start transcription.

There are other factors which influence how proteins interact with the DNA during transcription. DNA looping plays an important role in transcriptional control. This phenomena is mediated by binding of regulatory proteins to two distinct sites. The *gal* operon is one of the best characterized operons involving DNA looping. There are two operator sequences in *gal* operon, O_e and O_t. O_e is found upstream of two overlapping promoters and O_t is located downstream of the promoters (Irani et al., 1983; Adhya, 1987; Mandal et al., 1990). The loop structure is formed when a single repressor complex binds both operators and consequently RNA polymerase is constrained from binding to the structured promoters. An analogous mechanism has been observed in *lac* operon regulation. However, in the *lac* operon the operators are located downstream of the promoter (Mossing and Record, 1986; Kramer et al., 1987).

Finally, it has been noted that sequence-dependent DNA curvature affects many processes in which the DNA is structured, such as DNA bending, wrapping and looping. DNA curvature has been associated with many promoters.

**DNA curvature.** There are many unusual forms of DNA. Among those, intrinsic DNA curvature has been most studied the most and its biological significance has been shown. Intrinsic DNA curvature is a phenomena that plays an important role in a variety of DNA
transactions. While there are various methods for detecting altered DNA structure, the most sensitive and convenient method is polyacrylamide gel electrophoresis (Diekmann, 1987). Curved DNA tends to migrate more slowly in an acrylamide gel than non-curved DNA of equal length. Aberrant mobility is influenced by acrylamide concentration, temperature, and salt concentration in the gel (Diekmann and Wang, 1985; Dickmann, 1987).

Curved DNA was first identified in electrophoretic studies of kinetoplast minicircle DNA from *Leishmania tarantolata* (Marini et al., 1982). The first clue that kinetoplast DNA might be bent came from the observation that a restriction fragment from a *L. tarantolata* minicircle migrated anomalously slowly during electrophoresis on a polyacrylamide gel. It was shown that runs of adenine, or an adenine tract (A-tract), would produce a small bend in DNA helix. Intrinsically curved DNA is commonly characterized by runs of 4–6 adenine residues in phase with periodicity of B-form DNA. This periodicity places the A tracts on the same side of the helix and allows the angle of deflection from the helical axis resulting from each A tract to be additive, thus leading to a large overall bend in the DNA (Hagerman, 1985; Dickmann, 1986; Koo et al., 1986).

Several theoretical models have been proposed to explain intrinsic DNA bending on the molecular level. Among those, the first proposed model still is widely accepted. This model, called the “wedge model” (Trifonov and Sussman, 1980), assumes smooth global bending as a result of small additive wedges. The combination of tilt and roll cause formation of a wedge or angle between adjacent AT base pairs in the DNA helix (Trifonov and Sussman, 1980; Ulanovsky, 1987; Bolshoy et al., 1991). Such deformation in-phase with the helical repeat cause a long-range curvature. It has been recently proposed that the
DNA helix in an A-tract is characterized by base inclination in form of a negative roll (Haran et al., 1994). Progressive narrowing in the minor groove of the helix is the result of this negative roll.

In addition to A-tracks, there are other sequences which cause bending of DNA. Sequences with specific periodic dinucleotide, such as AG, CG, GA, or GC, have been shown to contribute in bending of DNA (Bolshoy et al., 1991). Compared to A-tracts, they bend the DNA to smaller degree (Fujimura, 1988; Milton et al., 1990; Bolshoy et al., 1991). Strong gel-mobility anomaly has been noticed for GGGCCC-containing DNA in the presence of divalent ions (Brukner et al., 1994). Perhaps the sequence dependent dynamic feature of DNA is influenced by metal ions. Another intrinsically curved sequence appears to be the GGCC element. This element is bent toward the major groove (Goodsell et al., 1993).

**DNA curvature in prokaryote promoters.** DNA curvature generally is generated by an A-tract sequence located upstream of the -35 region (Tanaka et al., 1991). Promoters containing this upstream sequence are a well documented phenomena in prokaryotes. It has been suggested that there is a relationship between intrinsic curved DNA and transcriptional activity in certain *E. coli* ribosomal and tRNA promoters (Nishi and Itoh, 1986; Bauer et al., 1988). Reduced activity has been observed when the curved DNA is deleted in several other promoters such as the *ompF* (Verda et al., 1981), the *bla* promoter from pUC19 (Ohyama et al., 1992), the *his* and 1PP promoters (Verda et al., 1981), and the Alu156 promoter from *B. subtilis* phage SP82 (McAllister and Achberger, 1988).
The effect of CRP in the activation of \textit{gal} promoter in \textit{E. coli} has been well documented. It has been shown that if the CRP binding site is replaced with synthetic or natural curved DNA, the transcriptional activity can be restored (Bracco et al., 1989). Similar results were reported using synthetic curved DNA in the \textit{lac} promoter (Gartenberg and Crothers, 1991). The addition of distamycin, a drug which is able to relax the DNA, to \textit{gal} p1 promoter, which has a curved sequence upstream of promoter, caused a significant reduction in transcriptional activity in the absence of the cAMP-CAP complex (Lavigne et al., 1992). These results suggest that similar to protein induced bending, sequence-specific DNA curvature enhances gene expression.

In studies from our laboratory, it was shown that sequence dependent DNA curvature immediately upstream of the -35 region can enhance RNA polymerase binding to promoters. Furthermore, it has been reported that deletion of curved DNA dramatically decreased promoter utilization by the RNA polymerase from \textit{B. subtilis in vivo} and \textit{in vitro}. It was also documented that one region of curved DNA will substitute for another when properly aligned, and the rotational orientation (on the helix) of the curve relative to the promoter was more important to function than the distance between the curved DNA and the promoter (McAllister and Achberger, 1988, 1989). Hybrid promoters were created by placing curved DNA from \textit{B. subtilis} bacteriophage promoters on \textit{E. coli} phage \textit{\lambda P_L} and \textit{P_R} promoters. The addition of curved DNA influenced the binding of the RNA polymerase from either \textit{B. subtilis} or \textit{E. coli} (McAllister, 1988). Wrapping of promoter DNA around the \textit{E. coli} RNA polymerase was documented for one series of these promoters (Nickerson and Achberger, 1995). \textit{B. subtilis} RNA polymerase also wrapped the DNA upstream of the
promoter around itself (Cheng, 1996). A model was proposed for all these findings. It was proposed that curved DNA enhances promoter function by facilitating the wrapping of the DNA around the RNA polymerase. This structured DNA-RNA polymerase complex allows the DNA helix to be untwisted and the two strands separated for transcription initiation.

The focus of this research was to test for the interaction of *E. coli* RNA polymerase with DNA upstream of the promoter consistent with DNA wrapping and to investigate the nature of this phenomenon. In this study, the Alu156 and Bal129 promoters from the *B. subtilis* bacteriophage SP82 were chosen for analysis. For each promoter, the nucleotide sequence upstream from -35 region contains intrinsic DNA curvature. A DNase I footprinting assay was used to study the interaction between promoter DNA and RNA polymerase. In addition, the effect of temperature, salt concentration, and heparin on RNA polymerase complex formation and wrapping was investigated. This study shows that *E. coli* RNA polymerase wraps the DNA upstream of the promoter around itself. A model for the nature of this interaction and its relation to the initiation of transcription is proposed.
MATERIALS AND METHODS

Materials. PCR reagents were supplied by Perkin Elmer. Restriction enzymes were purchased from either Bethesda Research Laboratories or New England Biolabs. DNase I was purchased from Boehringer Manheim GmbH. Permanganate was purchased from Sigma Chemical Company. The $[\gamma^{32}\text{P}]$ ATP and $[\alpha^{32}\text{P}]$ ATP were purchased from New England Nuclear, Dupont. All other materials used were of the highest quality available.

Plasmid transformation and isolation. *Escherichia coli* strain DH5a McR was grown in LB medium (Miller, 1972) at 37°C. *E. coli* cells were made competent using CaCl$_2$ washes (Lederberg and Cohen, 1974). Competent cells were mixed with 20-50 ng plasmid in the test tube, placed on ice for 20 minutes and heat shocked at 42°C for 2 minutes. The cells were cooled briefly on ice and diluted with L-broth followed by incubation at 37°C with shaking for 90 minutes. Between 0.2-1 ml of cells were concentrated and spread onto the agar plates containing 50 mg/ml ampicillin to select for plasmid containing cells. Selected colonies were isolated and cultured for plasmid isolation. Alkaline-lysis method of Birnboim and Doly (1979) was used in order to isolate plasmid DNA. To isolate highly purified plasmid DNA, large scale isolation was carried out followed by cesium chloride density gradient separation in the presence of ethidium bromide. The purified DNA was quantified by spectrophotometry.

Construction of Alu156 derivative promoters. An Alu156 promoter derivative which contain uncurved DNA upstream of -35 region was constructed. In order to disrupt the
intrinsic DNA curvature, Thymines were inserted into the middle of each A tract using site directed mutagenesis (Chen and Przybyla, 1994). In this method, two rounds of PCR were performed. In the first round, the AluUnc primer (5'-GCTAATATTCTGAATAATATTGCAATAAGTTGTTGAC-3') and the M13/pUC reverse sequencing primer (-48) 24-mer (New England Biolabs) were used to incorporate the mutations into the promoter. The template used in this round was plasmid pUC8 containing the original Alu156 promoter. The first PCR amplified fragment was gel purified and used directly as a primer together with the M13/pUC sequencing primer (-47) 24-mer (New England Biolabs) to direct a second round of DNA amplification using the Alu156 DNA template. Both rounds of PCR were performed in a Perkin Elmer Model 480 thermal cycle for 25 cycle at 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min. The products from the second round of amplification were digested with EcoRI and HindIII. The restriction fragments were then gel purified and ligated to pUC8 digested with the same enzymes. The promoter was named AluUnc.

To study the role of AT rich regions upstream of the promoter, two promoters in which AT rich DNA was substituted for the curved DNA were constructed using PCR. For the first promoter the AT-rich region immediately upstream of the E. coli rrnBP promoter, called the UP-element (Ross et al., 1993), was inserted upstream of -35 region. To construct this promoter, the M13/pUC sequencing primer (-47) 24-mer (New England Biolabs) and AluUp primer (5'-GCGAATTCAGAAAATTATTTTAAATTGTTGACTTCTCTACGAGGTTGAC-3') were used for DNA amplification with the plasmid pUC8 containing the wild-type Alu156 promoter as template. The amplified
products were digested with *EcoRI* and *HindIII* and gel purified. Purified fragments were ligated to pUC8 digested with the same enzymes. The promoter was named AluUp.

The second promoter contained 27 nucleotide long AT rich region upstream of the promoter. The same procedures were used except that the 27AT primer (5'-GCGAATTCTATATATAATTATATTCGTTGACTTT-3') was used instead of AluUp primer. This promoter was named Alu27AT. In order to replace the DNA upstream of the curved DNA in Alu27AT and AluUP with heterologous DNA in other promoters used in this study, *EcoRI* DNA fragment from AluExt promoter DNA was inserted at the *EcoRI* site of these promoter. Alu27At, AluUp, and AluUnc primers were synthesized in the Gene Lab, Louisiana State University.

To study the effect of AT-rich region, two other promoters were constructed one with a short stretch of AT and the other without any AT-rich region upstream of -35 region. For construction of the first promoter, AluExt which does not possess any curved DNA upstream of promoter was cut with *EcoRI*. The 5' extension was made flush using Klenow fragment and the gap was ligated. This promoter was named AT.

The last promoter lacked any AT-rich region upstream of promoter. In order to construct this promoter, after cutting the AluExt with *EcoRI*, the 5' over hang was digested with S1 nuclease to remove the single stranded DNA and then ligated. This promoter was named AluDel.

The promoter constructs were sequenced using the Circumvent Thermal Cycle Sequencing kit (New England Biolabs) to insure that there were no changes in the promoter.
sequence other than the intended mutations. Other promoters used in this study are listed in Table 1. All of these promoters are from our laboratory collection.

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Source</th>
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<tbody>
<tr>
<td>Alu 156</td>
<td>Early gene promoter from phage SP82 containing curved DNA, Alu156 is 82% homologous to <em>E. coli</em> promoter consenses sequence</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>AluExt</td>
<td>Alu156 derivatives in which the curved DNA was replaced by a fragment of pBR322, base pairs 376-467</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>Bal129</td>
<td><em>B. subtilis</em> bacteriophage SP82 promoter containing curved DNA, Bal129 is 90% homologous to <em>E. coli</em> promoter consenses sequence</td>
<td>Laboratory collection</td>
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<tr>
<td>BalExt</td>
<td>Bal129 derivatives in which the curved DNA was replaced by a fragment of pBR322, base pairs 376-467</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>λpR</td>
<td>Bacteriophage λ promoter</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>AluλpR</td>
<td>λpR derivatives in which curved DNA from Alu156 was inserted upstream of phage promoter</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>BalλpR</td>
<td>λpR derivatives in which curved DNA from Bal129 was inserted upstream of phage promoter</td>
<td>Laboratory collection</td>
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**Primer labeling.** Each primer was 5'end labeled using T4 polynucleotide kinase (New England Biolabs) and 30 mCi [g-32P ATP] (Dupont, New England Nuclear) at 37°C for 30 min. In each 25 ml reaction 20 pmol of primer and 10 units of kinase were used. The reaction was then denatured at 95°C for 5 min and stored at -20°C.

**PCR amplification.** Two primers, M13/pUC reverse sequencing primer (-48) 24-mer and the M13/pUC sequencing primer (-47) 24-mer (New England Biolabs) used for amplification. Only one of the two primers was labeled with [g-32P] ATP as it was.
Derivatives of pUC8 containing the various promoter constructs were used as templates in amplification reactions. Each reaction contained, 2.5 ml of supplied 10X PCR buffer, 2ml of a mixture containing 2.5 mM of each deoxynucleotide substrate, 2 ml 25 mM MgCl₂, 0.1 ml AmpliTaq DNA polymerase, 4ml 5'end-labeled primer (0.8 mM) and 1ml second primer (20 mM), 2 ml (0.2 ng/ml) DNA template and 11.5 ml water. PCR was performed in Perkin Elmer DNA Thermal Cycler Model 480 for 25 cycles of the following, 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min. Labeled products were purified through a Sephadex G-50 (Pharmacea Biotech) spin column (Neal and Florini, 1973).

RNA polymerase isolation. RNA polymerase was isolated from E. coli MRE600 as described by Spiegelman et al. (1978) with minor modifications (Achberger and Whitely, 1980; McAllister and Achberger, 1988). Sonication was used to lyse the cells, and RNA polymerase was purified by the sequential steps of polyethylene glycol-dextran phase partitioning, ammonium sulfate precipitation, gel filtration chromatography, and DNA cellulose chromatography. RNA polymerase activity was examined using an in vitro transcription assay (Spiegelman et al., 1978). SDS-PAGE electrophoresis was used to analyze subunit composition and purity of RNA polymerase (Lamml, 1970). The Bio-Rad protein assay was used to calculate RNA polymerase concentration. RNA polymerase was stored at -20°C in 35% glycerol.

DNase I footprinting analysis. To examine promoter-RNA polymerase interactions, a 190 μl of reaction mixture containing 40 mM Tris-HCl(pH 8.0), 10 mM MgCl₂, 50 mM NaCl, and 100,000cpm end-labeled DNA was incubated at 37°C with or without 2 mg RNA polymerase. After 5 min incubation, 0.04 μg of DNase I (Boehringer Manheim Corp.) was
added to mixture. Following a 30 sec digestion with DNase I, 20 µl of salt solution containing 3 M sodium acetate and 0.05 µg/µl yeast tRNA was added and two volumes of phenol/chloroform mixture were added to stop the reaction. After mixing with a vortex mixer, the phases were separated by centrifugation. The aqueous layer containing the DNA was ethanol precipitated, rinsed with 80% ethanol, and dried under reduced pressure. The DNA pellet was resuspended in 5 µl of loading buffer made with 10 ml deionized formamide, 10 mg xylene cyanol, 10 mg bromophenol blue, and 0.2 ml 0.5 M EDTA, pH 7.0. Samples were heated to 95°C for 5 min and immediately transferred to ice. DNA banding patterns was visualized by autoradiography after electrophoresing the samples on 6% polyacrylamide (acrylamide to bisacrylamide, 30:1.5) gel containing 7M urea in TBE buffer at 1700 volts. Following electrophoresis the dried gels were analyzed by autoradiography.

**Gel retardation analysis.** To analyze the relative affinity of *E. coli* RNA polymerase for different promoters, gel retardation analysis was performed as described by Ausubel et al., 1989 with minor modifications. End-labeled DNA fragments were incubated with or without RNA polymerase in buffer containing 40 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, 50 mM NaCl and 1 µg of non-specific competitor DNA at 37°C for 10 min. Following the addition of 4 µl of loading dye (26% Ficoll, 0.1% Bromophenol blue), DNA-RNA polymerase complexes were resolved in 4% polyacrylamide gel (acrylamide to bisacrylamide, 30:2) with high ionic strength buffer (50 mM Tris, 400 mM Glycine, and 2 mM EDTA pH 8.5). The gel was electrophoresed at 150 volts at room temperature for
two hours. Then gel was transferred to Whatman 3 MM paper, dried and autoradiographed. Both free and RNA polymerase bound DNA were quantified by densitometry of autoradiograms.

**KMnO₄ footprinting.** To detect open promoter complex formation, the basic reaction conditions were the same used for DNase I footprinting. After 5 min incubation at 37°C with or without RNA polymerase, 5 μl of 80 mM KMnO₄ was added to the mixture. The reactions were stopped by adding 10 μl 2-mercaptoethanol and 15 μl of 3.0 M sodium acetate after 5 min. The samples were extracted with two volume of a phenol/chloroform mixture and precipitated with ethanol. DNA pellets were washed with 80% ethanol, dried, and resuspended in 5 μl formamide loading buffer as described before. After heating the samples to 95°C for 5 min, they were analyzed by polyacrylamide gel electrophoresis as described for DNase I footprinting. Following electrophoresis the dried gels were analyzed by autoradiography.

**Single-round run-off transcription assay.** All DNA templates used for the experiment were synthesized by PCR amplification. For each reaction, an equal amount of DNA template was used as determined by densitometry of ethidium bromide stained polyacrylamide gels. In addition to template DNA, each reaction contained 0.5 μl of *E. coli* RNA polymerase (1.5 mg/ml), 2 μl of 10X transcription buffer (400 mM Tris-HCl, 100 mM MgCl₂, 500 mM NaCl₂), and water for the total reaction volume of 17.5 μl. The reactions were mixed on ice and then transferred in duplicate to desired temperature for 5 min. Then 2 μl of nucleotides (20 mM GTP, 20 mM CTP, 20 mM UTP, 4 mM ATP, and 0.5 mCi/ml, 3000 Ci/mmol [α³²P]ATP) and 1 μl of heparin (1 mg/ml) was added to each reaction at the
same time. Half of the reactions were shifted to 37°C after 30 second and the rest remained at incubated temperature. After 5 min., all of the reactions were stopped by adding urea to a final concentration of 5M.

Transcription products were analyzed by electrophoresing the samples on 6% polyacrylamide (acylamide to bisacrylamide, 30:1.5) gel containing 7M urea in TBE buffer at 500 volts. After electrophoresis, the gels were dried and exposed to X-Ray film (Kodak, X-OMAT). Resulting bands were quantified by densitometry.
RESULTS

The present work focuses on the role of intrinsic DNA curvature upstream of -35 promoter region in DNA wrapping and investigates the nature of interaction between *E. coli* RNA polymerase and curved DNA. The Alu156 and Bal129 promoters from the *B. subtilis* bacteriophage SP82 are 83% and 90% homologous to the consensus sequence of *E. coli* promoters, respectively. These two promoters are recognized by *E. coli* RNA polymerase and both have intrinsically curved DNA sequences upstream of -35 region (Figure 1). Previous studies have demonstrated that upstream DNA curvature is required for efficient utilization of these promoters by *B. subtilis* RNA polymerase (McAllister and Achberger, 1988:1989). Curved DNA also appears to play a role in DNA structuring by *E. coli* RNA polymerase. When curved DNA was spliced onto the phage λP_R promoter, *E. coli* RNA polymerase wrapped the curved DNA around the enzyme (Nickerson and Achberger, 1995). To study the phenomena of DNA wrapping, the Alu156 and Bal129 promoters and their derivatives have been used in this study.

**DNase I footprint analysis of the interaction between *E. coli* RNA polymerase and Alu156 and Bal129 and their derivatives.** It was of interest to determine if DNA wrapping could occur in promoters other than those used in a previous study in our laboratory. To investigate this problem, Alu156 and Bal129 and their derivatives was used. The AluExt and BalExt are derivatives of Alu156 and Bal129, respectively, in which the curved DNA upstream of promoter was replaced by DNA with no curvature (Figure 2). The DNase I footprint analysis on both strands of all these promoters was performed. This
Figure 1: Nucleotide sequence of the Alu156 and Bal129 promoters. Bases representing the +1 transcription start site, the -35 and the -10 regions are in bold type. The runs of adenine in the DNA upstream of the -35 region are underlined.
Figure 2: Nucleotide sequence of the Alu156, Bal129, and their derivatives. Bases representing the +1 transcription start, the -35 and -10 regions are in bold type. The runs of adenine in the DNA upstream of the -35 region are underlined. Nucleotide sequence downstream of the -35 region is the same for each wild-type promoters and its derivative.
assay can locate the protein binding site. Bound protein will protect the DNA from cleavage with DNase I. DNase I cutting is also sensitive to protein-induced changes in the DNA conformation. DNA conformation changes are often observed as DNase I hypersensitive sites. DNase I digestion pattern indicative of wrapping is identified as a series of enhanced cleavages (dark bands) followed by protection (light bands) with a 10 base pair periodicity. Figure 3 corresponds to an autoradiogram after DNase I footprint analysis of both strands of Alu156. Lanes marked by a minus sign are control reactions where the DNA fragment containing the promoter was digested with DNase I in the absence of RNA polymerase. When RNA polymerase was bound to the Alu156 promoter (lanes marked by plus sign), a large protection area typical of results with prokaryote RNA polymerases was observed from +24 to -55 in lower strand of DNA (Figure 3A). The nucleotides are numbered relative to the transcription start site at position +1. Hypersensitive sites, represented by dark bands relative to control lane, were observed at -58, -59, -68, -69, -71, -92, -97, -101, -102, and from -108 to -110. The curved DNA region from -40 to -65 is weakly cleaved by DNase I because of the altered DNA conformation in this region. Small regions of protection from DNase I were observed following these hypersensitive areas. For the upper strand of this promoter, the pronounced protected region was observed from +21 to -56 (Figure 3B). This region was interrupted with hypersensitive sites at -24, -44, and -47. DNase I hypersensitive area upstream of promoter were around -57, -58, -59, -60, -67, -68, -78, -79, -80, -81, -86, -87, -98, -99, -112 and -113 followed by protected regions. This pattern of alternating protection and enhanced cleavages with 10 base pair periodicity in both strands of Alu156 was indicative of wrapping.
Figure 3: DNase I footprint analysis of the Alu156 promoter. Panel A represents the footprinting of the lower (template) strand and panel B correspond the upper strand DNA footprint. DNase I digestion patterns in the absence (lanes with minus sign) and presence (lanes with plus sign) of RNA polymerase are shown. The bands are numbered relative to the transcription start site at position +1.
The DNase I pattern for AluExt, which lacks the curved DNA, was similar to that of Alu156 promoter in the protected region from DNase I cleavage from +24 to -46 in lower strand (Figure 4A) and +20 to -56 in upper strand (Figure 4B). Enhanced cleavages were observed around the -60 region of both strands. Evidence of protein-DNA interaction consistent with DNA wrapping was less obvious for this promoter. Figure 5 represents the summary of the DNase I footprinting results on both strands of Alu156 and AluExt promoters compiled from three separate experiments.

Analysis of *E. coli* RNA polymerase binding to the Bal129 promoter has also provided evidence for DNA wrapping. The footprint for lower strand of Bal129 extended from +17 to -47, which was typical for other *E. coli* promoters (Figure 6A). Enhanced cleavages upstream of promoter were observed at -49, -48, -58, -59, -60, -69, -70, -71, -73 and -93, and -94. For upper strand of Bal129 (Figure 6B) the footprint region was from +20 to -43 with enhanced cleavages at -23 and a gap at -21, and -22. Hypersensitive areas to DNase I were observed at -44 to -46, and -53. Protected regions were observed around -50, -60, and -70. For technical reasons, footprint data upstream of -72 was unavailable for the upper strand. This pattern of DNase I cleavage for both strands of Bal129 was in agreement with presence of DNA wrapping.

The general footprint for BalExt was the same as Bal129 promoter downstream of -40 except enhancement cleavages at -32, and -37 to -39 in upper strand and -37 in lower strand (Figure 7). Almost no upstream interaction was detected for this promoter. Evidence of DNA wrapping for BalExt was not obvious. This suggests that the curved
Figure 4: DNase I footprinting of the AluExt promoter bound by RNA polymerase. Footprint of the lower (template) strand of DNA is shown in panel A and panel B represents the footprinting of upper strand of the promoter. Presence and absence of RNA polymerase are indicated by plus and minus signs, respectively. The DNA bands are numbered relative to the transcription start site at position +1.
Figure 5: The summary of DNase I footprint analysis on both strands of the Alu156 (A) and the AluExt (B) promoters. The -10 and -35 regions are in bold type. The line over the upper strands and below the lower strands represent the area protected from DNase I cleavage. Hypersensitive regions are marked by arrows. The results were compiled from three separate experiments.
A

-120 -110 -100 -90 -80 -70

GAAAGATATCCCTACAGCAGATGGACAGGATCCGGGGAATTCTCCTGCGATCCTGCCTAGGCCCCTTAAG

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

AAAATCTCCTGAAAAATTGTGTTGACTTTCTCTACGAGGTGTTGGCATATAATATTCTTA
TTTTAGGACATTAAAAACGTAAAACTGCTCACAACCCTATTAATAGAAT

+10 +20 +30

ACAACACGAGACGCTAGGACGGATCCGGGGAATTCTCCTGCGATCCTGCCTAGGCCCCTTAAG

B

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

TCACCGATGGAAGATCCCGGCAATTCGTGACTTTCTCTACGAGGTGTTGGCATATAATATTTTA
AGTGGCTACCCCCTTTCTAGGGCCCCCTTAAGCAACTGAAAGAGATGCTCCACACCGTATTATTAGAAT

+10 +20 +30

ACAACACGAGACGCTAGGACGGATCCGGGGAATTCTCCTGCGATCCTGCCTAGGCCCCTTAAG
Figure 6: DNase I footprint analysis of Ball29 promoter. Lower (template) strand (panel A) and upper strand (panel B) of the promoter are footprinted in the absence (lanes with a minus sign) and presence (lanes with a plus sign) of RNA polymerase. The DNA bands are numbered relative to transcription start site at +1.
Figure 7: DNase I footprint analysis of BalExt promoter. Lower (template) strand (panel A) and upper strand (panel B) of the promoter are footprinted in the absence (lanes with a minus sign) and presence (lanes with a plus sign) of RNA polymerase. The DNA bands are numbered relative to transcription start site at +1.
DNA contributes to the interaction of RNA polymerase with DNA upstream of the -35 region. Figure 8 represents the summary of footprint analysis for both strands of Bal129 and BalExt.

There is an excellent agreement between the results for both promoters. DNase I digestion pattern indicative of DNA wrapping was more obvious for both wild-type promoters relative to the altered promoter lacking curved DNA. There was a periodicity of approximately 10 base pairs in the pattern of enhanced cleavages and protection in both wild-type promoters. This pattern provided evidence that the upstream curvature of Alu156 and Bal129 was bent when wrapped around the RNA polymerase. It should be noted that the possibility of second RNA polymerase binding to upstream region was ruled out by footprinting the promoters at different RNA polymerase concentrations. At the lowest concentration of RNA polymerase where binding to the promoter was observed, wrapping was present. The DNase I pattern indicative of wrapping disappeared at the same point as the main footprint (+20 to -50 region). This indicates that wrapping was not the result of binding of another RNA polymerase to upstream sequence.

Nature of the interaction between RNA polymerase and DNA upstream of the -35 region. Electrostatic interactions between RNA polymerase and the backbone of DNA are essential for sequence-independent DNA binding. Salts such as NaCl affect sequence-independent binding between RNA polymerase and promoter DNA by competing with RNA polymerase for charged phosphate residues on the DNA backbone (Roe and Record, 1985; Suh et al., 1992). To explore the nature of interaction between RNA polymerase and DNA upstream of the curved DNA, NaCl was used to disrupt electrostatic interactions. DNase
Figure 8: The summary of DNase I footprinting results on both strands of Bal129 (A) and BalExt (B). The -10 and -35 regions are in bold type. The lines over the upper strand and below the lower strand represent the area protected from DNase I cleavage. Hypersensitive regions are marked by arrows. The results were compiled from at least two experiments for each strand.
I footprinting analysis was performed for both wild-type promoters and their derivatives using binding buffer containing various concentrations of NaCl. Since the results of DNase I footprinting analysis were in agreement for both strands of each promoter, only the interactions with lower strands were analyzed in this study.

Figure 9 demonstrates the effect of different salt concentration on DNA wrapping for the lower strand of Alu156. Lane 1 and 7 represent control reactions which DNase I footprinting was performed in the absence of RNA polymerase. In lanes 2 to 6, salt concentrations were incrementally increased from 50 mM to 200 mM, respectively. The major footprint area (i.e., +20 to -56) consistent with the RNA polymerase bound to the promoter, was observed at every salt concentration tested. Some differences were detected among various salt concentrations. For example, bases -38 and -58 were not protected from DNase I at salt concentrations greater than 100 mM. This indicates that RNA polymerase-DNA complexes formed at lower salt concentrations were different from those at higher salt concentrations. Evidence of DNA wrapping progressively faded with increasing NaCl concentration. Visible changes were observed at different regions upstream of promoter. For example, enhanced cleavages at -61, and -62 disappeared by increasing the salt concentration to 100 mM. Obvious differences were displayed at the regions around -70, -80, -90, -100, -110, and -120. Even bases very far upstream exhibit evident differences among various salt concentration. Basically, enhanced cleavages and protection areas indicative of DNA wrapping were lost gradually by increasing the salt concentration in binding buffer.
Figure 9: DNase I footprint analysis of the Alu156 promoter bound to RNA polymerase as a function of NaCl concentration. Lanes 1 and 7 represent the DNase I digestion pattern in the absence of RNA polymerase at 50 and 200 mM NaCl concentration. Lanes 2 to 6 correspond to the footprint of the promoter bound by RNA polymerase at 50, 75, 100, 150, and 200 mM NaCl respectively.
The AluExt promoter was also footprinted as a function of salt concentration (Figure 10). Footprint area from +20 to -53 was observed in all lanes in Figure 10 except two control lanes. Minor protection around -50 and enhanced cleavages around -60 disappeared as the salt concentration increased. Even in the absence of detectable DNA wrapping, the RNA polymerase-promoter complex that predominate at 50 mM NaCl differed from those at 150 to 250 mM.

Effect of salt concentration on Ball29 promoter was also examined (Figure 11). As observed for last two promoters, the large footprint area from +19 to -50 remained the same at all salt concentrations except for changes at -40 and -48 regions consistent with the presence of different complexes at various salt concentrations. Upstream interactions indicative of wrapping were diminished by increasing the salt concentration. Enhanced cleavages around -60, -71, and -91 disappeared at lane 5, which represents 150mM NaCl concentration. Protected areas around -50, -60, -80, and -100 were diminished at the same salt concentration. At 150 mM NaCl or greater, no changes in the DNase I pattern were observed upstream of -59.

In the case of the BalExt promoter, as observed for AluExt, the limits of the footprint at the promoter region were similar at all salt concentrations (Figure 12). Minor changes observed upstream of promoter around -60 were diminished when salt concentration was increased. For each promoter tested, protection at +16 to +20 increased with increase of NaCl concentration.
Figure 10: Effect of NaCl concentration on the DNase I footprint pattern of AluExt. DNase I digestion patterns in the absence of RNA polymerase at 50 and 250 mM NaCl concentration are shown in lanes 1 and 8. Lanes 2 to 7 correspond to the footprint of the promoter at 50, 75, 100, 150, 200, and 250 mM NaCl, respectively.
Figure 11: DNase I footprint analysis of the Ball29 promoter as a function of NaCl concentration. DNase I digestion patterns in the absence of RNA polymerase at 50 and 250 mM NaCl concentration are shown in lanes 1 and 8. Lanes 2 to 7 correspond to the footprint of the promoter at 50, 75, 100, 150, 200, and 250 mM NaCl, respectively.
Figure 12: Effect of NaCl concentration on the DNase I footprint analysis of the BalExt promoter. DNase I digestion pattern in the absence of RNA polymerase at 50 and 250 mM NaCl concentration are shown in lanes 1 and 8. Lanes 2 to 7 correspond to the footprint of the promoter at 50, 75, 100, 150, 200, and 250 mM NaCl.
In general, as the salt concentration increased, wrapping decreased. DNase I digestion patterns consistent with the RNA polymerase bound to the promoter were observed at every NaCl concentration tested. Based on our results it can be concluded that wrapping is more favorable at low salt concentration suggesting the electrostatic nature of the phenomenon. This would be expected if these DNA-RNA polymerase interactions were sequence independent.

The relationship between open promoter complex formation and DNA wrapping. Potassium permanganate was used to probe the RNA polymerase-promoter complexes formed at various NaCl concentration for both Alul56 and Ball29 (Figure 13). Potassium permanganate is an oxidizing agent, which preferentially nicks at T and C residues in single-stranded DNA (Sasse-Dwight and Gralla, 1988). It was noted that while open promoter complexes decreased at high salt concentration (i.e., 200 mM), they were still observed. DNA wrapping as visualized by DNase I footprinting was lost at high NaCl concentrations. This indicates that there are open complexes in which the DNA is no longer wrapped.

Test for DNA wrapping at different stages of transcription initiation. It is known that at low temperature, the conformational changes leading to the open promoter complex do not occur (Cowing et al., 1989; Mecsas et al., 1991). When Alul56 was tested for open promoter complex formation at different temperatures, it was observed that at 0°C almost no open complexes were formed (Figure 14). Upon raising the temperature, significant increase in the amount of open promoter complex formation was observed. RNA polymerase-promoter complexes at 0°, 5°, 15°, 22°, and 37°C were examined by DNase I footprinting (Figure 15). The results were consistent with the presence of DNA wrapping.
Figure 13: Open promoter complex formation at different salt concentrations for Bal129 (A) and Alu156 (B). Potassium permanganate probe was used to detect open promoter complexes formed in the absence (lane with minus sign) and presence of RNA polymerase. The salt concentration used in each reaction is marked above the lines. The sequence of the open promoter complex regions are marked.
A.

| NaCl (mM) | 1 | 50 | 75 | 100 | 125 | 150 | 200 |

B.
Figure 14: Effect of temperature on open promoter complex formation for the Alu156 promoter. Absence and presence of RNA polymerase in each reaction are shown by minus and plus sign respectively. The temperature at which each reaction was performed is shown. The sequence of open complex region is marked.
Figure 15: DNase I footprint analysis of Alu156 as a function of temperature. Presence or absence of RNA polymerase is represented by plus and minus sign. The temperature of each reaction is shown.
of all temperatures. Basically, the protected region indicative of sequence-specific RNA polymerase-promoter interaction was detected at every temperature. Wrapping occurred at low temperature in the absence of open promoter complexes. RNA polymerase footprint was shorter at 0°C. Protection from the +1 region to +20 is significantly reduced at 0°C. This is a signature footprint for closed promoter complexes.

To confirm the presence of wrapped closed complexes, heparin was used in DNase I footprinting analysis. Heparin is strong DNA competitor that rapidly binds free RNA polymerase but not enzyme stably bound to the DNA or enzyme engaged in RNA synthesis (Walter et al., 1967). Closed complexes with short half lives are sensitive to effect of heparin (i.e., they irreversibly dissociate in the presence of heparin). The Alu156 promoter was footprinted at 37°C and 0°C (Figure 16) with and without a heparin challenge. RNA polymerase was allowed to bind the Alu156 promoter at the indicated temperature. To one sample at each temperature, heparin was added for 30 second prior to DNase I treatment. At 37°C, addition of heparin virtually eliminated the DNase I pattern consistent with DNA wrapping. Since heparin eliminates sensitive closed promoter complexes, this indicated that unwrapped heparin resistant open complexes were formed at 37°C. In addition, wrapped complexes appeared to be closed complexes. It was previously demonstrated that at 0°C almost no open promoter complexes were formed. DNase I digestion pattern consistent with wrapping was observed in the absence of heparin at 0°C. Upon addition of heparin, in addition to loss of wrapping, the footprint was diminished too. This suggests that all wrapped complexes were closed complexes.
Figure 16: Effect of heparin on the footprint analysis of the Alu156 promoter bound to RNA polymerase at 37°C and 0°C. The minus sign represents the DNase I digestion pattern in the absence of RNA polymerase. Lane marked with the plus sign (+) represent the binding of RNA polymerase without the heparin challenge. Lanes in which the reactions were challenged with heparin are designated by “+H”.

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Effect of AT-rich regions in DNA wrapping and transcription efficiency. The α subunit of RNA polymerase is known to bind an AT-rich sequence upstream of the -35 region (Ross et al., 1991). Since curved DNA is AT rich, the contribution of the AT-rich sequence was examined. To compare the role of AT-rich region upstream of promoter and curved DNA in DNA wrapping and transcription efficiency, five different promoter-containing fragments were constructed. In one promoter, AluUp, the DNA upstream of -35 region in AluExt promoter was replaced with the α subunit binding site, the so called “UP element”, of *E. coli* ribosomal RNA promoter *rrnB* P1. In second promoter, AluUnc, a non-curved AT-rich region was constructed by insertion of T’s in the middle of each A-tract upstream of Alu156 promoter. These mutations eliminate curvature while preserving the AT-rich nature of this promoter. Both Alu8AT and Alu27AT promoters contained AT-rich regions upstream of the -35 region of AluExt promoter. However, the length of this region in Alu8AT was shorter than that of Alu27AT. The AluDel promoter was constructed as a control with no AT-rich region upstream of AluExt promoter. The relevant nucleotide sequences of these promoters are listed in Figure 17 in comparison with the sequence of the Alu156 and AluExt promoters.

To examine the specific interactions between *E. coli* RNA polymerase and the AT-rich promoters, DNase I footprinting analysis was performed. This approach was used to determine the role of AT-rich region in DNA wrapping. Each promoter sequence was digested with DNase I in the absence and presence of RNA polymerase. Figures 18 and 19 illustrate the DNase I digestion patterns for the lower strands of each promoter. The DNase I digestion pattern indicative of wrapping that was observed for Alu156 was reduced in the
Figure 17: Nucleotide sequence of the Alu156, AluExt, AluDel, and AT-rich derivatives. AT-rich regions are underlined. The -10, -35, and +1 regions are in bold type. An asterisk is placed under the bases in AluUnc that differ from Alu156.
Figure 18: DNase I footprint analysis of the Alu156, AluUnc, AluUp, and Alu27AT promoters. Lanes depicting DNase I digestion in the presence and absence of RNA polymerase are indicated by plus and minus sign. The DNA bands are numbered relative to the transcription start site at position +1.
Figure 19: DNase I footprint analysis of the Alu156, AluExt, Alu8AT, and ATDel promoters. DNase I patterns generated in the presence and absence of RNA polymerase are indicated by plus and minus sign. The DNA bands are numbered relative to the transcription start site at position +1.
AluUnc promoter (Figure 18). For the rest of promoters, evidence of protein-DNA interactions consistent with DNA wrapping were not obvious. 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 +20 to -57. The AluUp promoter displayed a strongly protected region between -37 and -58 corresponding to the UP element. The AluExt and AluDel, which lacked AT-rich regions and curved DNA, displayed no significant DNA wrapping. Likewise, DNA wrapping was not observed for Alu8AT promoter (Figure 19).

Gel retardation analysis was performed to determine the relative binding affinity of the RNA polymerase for the original Alu156 and each of the promoters listed in Figure 17. The binding reactions were carried out in binding buffer with equivalent amount of 32P end-labeled promoter. For each concentration of RNA polymerase used, the DNA fragment containing the Alu156 promoter was efficiently bound by E. coli RNA polymerase (Figure 20 and 21). This suggest that RNA polymerase binding is enhanced by DNA curvature upstream of promoter. An unexpected result was observed for Alu27AT promoter. RNA polymerase displayed a dramatically low affinity for this promoter.

In order to compare the promoter strength in vitro, the single-round run-off transcription assay was performed. This assay measures the number of the transcribable complexes at the time of assay. Equal amount of DNA for each promoter was incubated in the presence of RNA polymerase at each temperature tested. Heparin and mixture of all four nucleotides triphosphates including [α 32P] ATP were added to each reaction. After 10 minutes, the reactions were stopped by adding urea and heating to 95°C for 5 minutes, the sample were analyzed by polyacrylamide gel electrophoresis. The relative amount of
Figure 20: Gel retardation analysis of Alu156, AluUnc, AluUp, and Alu27AT. Equivalent amounts of $^{32}$P end-labeled DNA fragments were incubated with various amounts of RNA polymerase at 37°C for 10 minutes, followed by electrophoresis. Lanes marked with a minus sign represent the absence of RNA polymerase. The RNA polymerase concentration was increased as it is marked by the symbol. The RNA polymerase amounts were 0.005, 0.016, and 0.05 μg. The DNA bands representing the RNA polymerase bound complexes and free DNA are marked.
Figure 21: Gel retardation analysis of Alu156, AluExt, Alu8AT, and AluDel. Equivalent amounts of $^{32}$P end-labeled DNA fragments were incubated with various amounts of RNA polymerase at 37°C for 10 minutes, followed by electrophoresis. Lanes marked with a minus sign represent the absence of RNA polymerase. The RNA polymerase concentration was increased as it is marked by the symbol. The RNA polymerase amounts were 0.02, 0.07, and 0.2 μg. The DNA bands representing the RNA polymerase bound complexes and free DNA are marked.
Figure 22: Transcribable promoter complexes formed as a function of temperature. Relative number of transcripts for Alu156(●), AluExt(□), AluUnc(▲), AluUp(▼), and Alu27AT(●) without (A) and with (B) temperature shift were determined by densitometry from an autoradiograph of a polyacrylamide gel containing 32P labeled transcripts. Both panels contain representative data for single-round, run-off transcription in the presence of heparin.
transcripts formed for each promoter was determined by densitometry. The results are shown in Figure 22A. As expected, very little transcription was observed at 0°C, with increased temperature, transcription increased for all promoters. At low temperature, the Alu156 displayed slightly better transcription relative to the other promoters. The Alu27AT displayed very low transcription at all temperatures. For second set of reactions, 30 seconds after addition of heparin and nucleotides, they were transferred to 37°C for 10 minutes prior to stopping the reactions. This temperature shift allows the heparin resistance complexes to form open complexes and start transcription. The relative number of transcripts as a function of temperature are shown in Figure 22B. The Alu27AT was the weakest promoter in all temperatures. This result confirms the result from gel retardation assay where RNA polymerase exhibited a very low affinity for this promoter. In the temperature shift reactions, significant transcription at low temperature was observed for the original Alu156 promoter in contrast to the results observed for panel A, where small number of transcripts where formed even at 10°C for all promoters tested. At temperatures above 30°C no significant difference was observed among promoters (data not shown). This indicates that the step in transcription initiation aided by curved DNA is normally inefficient at low temperatures. The presence of curved DNA stimulates the formation of a heparin resistant, closed complex. In the single round transcription assay, open complex formation (i.e., strand separation) appears to be the overall rate limiting step for Alu156 and its derivatives.

Effect of a mutation in the C-terminal domain of α subunit on the interaction between RNA polymerase and DNA upstream of the -35 region. To study the role of α subunit in the DNA wrapping, RNA polymerase with a deletion starting at position 235 of C-
terminal domain (CTD) of α subunit was used. RNA polymerase with and without the CTD mutation were a gracious gift from Dr. Richard Gourse at University of Wisconsin-Madison. It has been shown that protection of UP element DNA by the CTD mutant RNA polymerase is severely reduced in footprinting experiment (Ross et al., 1993). Three hybrid promoters AluPr, BalPr, and λP R (McAllister, 1988) were used in footprinting study with mutant RNA polymerase. The phenomenon of DNA wrapping has been documented for both AluPr and BalPr (Nickerson and Achberger, 1995). These two promoters contain the curved region of Alu156 and Bal129 upstream of λP R promoter. λP R lacks the curved DNA immediately upstream of the promoter and no obvious evidence of wrapping was observed for this promoter.

The results of footprinting with the mutant RNA polymerase are illustrated in Figure 23. For each promoter, the DNase I digestion pattern in the absence of RNA polymerase is shown in lane 1. Lane 2 represents the footprint of each promoter using wild-type RNA polymerase and lane 3 shows the footprinting analysis using the CTD mutant RNA polymerase. Evidence of DNA wrapping was observed for AluPr and BalPr when wild-type RNA polymerase was used. For λP R no obvious wrapping was observed. This result was in agreement with published data. Addition of mutant RNA polymerase to λP R did not change the pattern except that an enhanced region was observed around -50 region, which is part of α binding site. Since mutation in α prevents binding of the subunit to this region, mutant RNA polymerase can not protect this region from DNase I cutting. This “α signature” was observed for other two promoters as well. DNA wrapping was diminished
Figure 23: DNase I footprint analysis of the AluP_R, BalP_R, and λP_R promoters as a function of wild-type (lane 2) and mutant (lane 3) RNA polymerase. The nucleotide are numbered approximately relative to transcription start site at position +1.
for both AluP_r and BalP_r when mutant RNA polymerase was used. This indicates that the curved DNA is an α binding site and binding of α subunit to its binding site aids DNA wrapping in the promoters containing curved DNA.
DISCUSSION

Effect of DNA curvature immediately upstream of the -35 region on DNA wrapping by the RNA polymerase. In a previous study using hybrid promoters containing curved DNA, *E. coli* RNA polymerase was shown to wrap the curved DNA around the enzyme (Nickerson and Achberger, 1995). The hybrid promoters were made by splicing the curved DNA found upstream of the Alu156 and Ball29 promoters of *B. subtilis* phage SP82 onto λPr promoter (McAllister, 1988). In this limited study, the phenomenon of wrapping was examined on one strand of those promoters. One of the first questions asked in the present study was whether DNA wrapping could be observed for other promoters containing curved DNA. To explore this question, the Alu156 and Ball29 promoters were used. Both of these promoters were isolated from *B. subtilis* bacteriophage SP82 and contain curved DNA immediately upstream of -35 region. DNase I footprinting was performed to investigate the interaction between the *E. coli* RNA polymerase and these promoters. Large region of protection demonstrating the tight binding of RNA polymerase to -10 and -35 regions was observed for both promoters. The DNase I footprint patterns for these two promoters exhibited enhanced cleavages and sites protected from DNase I repeated almost every 10 base pair in the DNA upstream of promoters. This pattern was observed for both strands of both promoters. This feature is indicative of DNA wrapping around RNA polymerase. A similar pattern was observed when DNA is wrapped around proteins such as nucleosomes (Shaw et al., 1976; Prunell et al., 1984). The DNase I patterns of both strands combine to describe interactions characterized by region of DNA wrapped around
the enzyme and larger region of protection from -70 to -90 consistent with the DNA bound within a fold or cleft of the RNA polymerase. For derivatives of Alu156 and Bal129 which lack curved DNA, the footprint of the promoter region was the same as wild-type promoters. However, there was no obvious evidence of the DNase I cleavage pattern consistent with DNA wrapping upstream of these promoters. This indicates that curved DNA facilitated the wrapping of DNA upstream of the promoter around the RNA polymerase. Similar results were observed using the RNA polymerase from *B. subtilis* (Cheng, 1996).

**Model for the role of DNA curvature in RNA polymerase binding and transcription initiation.** Sequence-directed and protein-induced DNA curvature has been found in promoters of both prokaryotes and eukaryotes. It has been documented that intrinsically bent DNA plays a role in modulation of transcription initiation (Lamond and Travers, 1983; Bossi and Smith, 1984; Gourse et al., 1986; McAllister and Achberger, 1988). Based on previous studies in our laboratory, a model was proposed for the role of curved DNA in transcription initiation. It was proposed that curved DNA enhances promoter function by facilitating the wrapping of the DNA around the RNA polymerase. The structured DNA-RNA polymerase complex allows the DNA helix to be untwisted and the two strands separated for transcription initiation.

The model addressed the fact that there are multiple RNA polymerase promoter complexes identified with conformational changes in the DNA and enzyme. Among these complexes are the initial closed RNA polymerase-promoter complexes (RP_CI), a second closed complex in which the strands of the DNA have been partially untwisted but remain...
base paired (RP\(_{\infty}\)), and the open promoter complex in which DNA strand separation has occurred between the -10 region and +1 start site (RP\(_{\infty}\)). Previous studies with \textit{B. subtilis} RNA polymerase have demonstrated that curved DNA associated with promoters aids binding and the formation of open promoter complex (McAllister and Achberger, 1988; Stemke, 1993). More recently DNase I footprinting studies with \textit{B. subtilis} RNA-polymerase demonstrated the presence of a closed complex in which the upstream DNA is wrapped around the RNA polymerase (Cheng, 1996). This suggested that curved DNA stimulates a step prior to RP\(_{\infty}\). Prior to the present study, little evidence, other than the existence of DNA wrapping for hybrid promoters, was available to address this model to the \textit{E. coli} RNA polymerase.

The model proposed in our laboratory is similar to a model proposed for the role of \textit{E. coli} CRP, a DNA bending regulatory protein, in transcription activation. It has been demonstrated that curved DNA can replace the CRP binding site for the \textit{lacP1} promoter and \textit{galP1} promoters (Barco et al., 1989; Gartenberg and Crothers, 1991). Based on the model proposed by Gartenberg and Crothers (1991), curved DNA localizes at the end of a superhelical domain and facilitates the wrapping of DNA around RNA polymerase bound to the promoter. This structure favors the transcription initiation.

Based on present the study we were able to relate \textit{E. coli} RNA polymerase to the existing model and advance it. The presence of an additional closed promoter complex between the initial closed complex and the open promoter complex has been identified in many studies (Kadesch et al. 1982; Buc and McClure, 1985; Straney and Crothers, 1985). The RP\(_{\infty}\) is stable at low temperature (i.e., the predominant complex formed below 20°C)
and resistant to RNA polymerase competitors such as heparin and poly d(AT) (Buc and McClure, 1985; Spascky et al. 1985; Schickor et al., 1990). The transition step from RP$_C$ to RP$_O$ became the rate limiting step below 20°C (Buc and McClure, 1985; Spassky et al. 1985). We now have evidence that the presence of curved DNA affects the formation of RP$_C$. A single-round transcription assay was used in a temperature shift format. RNA polymerase was allowed to form complexes with various promoters at 0°, 10°, 20°, and 30°C prior to the addition of a mixture of the four nucleotide triphosphates and heparin. The nucleotides allow RNA syntheses from RP$_O$, and the heparin binds free RNA polymerase. RP$_C$ is said to be heparin sensitive since it dissociates rapidly and free RNA polymerase is bound by heparin. If the reactions were shifted to 37°C after addition of heparin and nucleotides, transcription from the low temperature complexes increased dramatically for Alu156 relative to promoters without curved DNA. Curved DNA on Alu156 allowed formation of RP$_C$ at low temperatures (0° and 10°C) which quickly isomerized to RP$_O$ at 37°C. Taking this result in consideration, our updated model proposes that by facilitating the wrapping of DNA around RNA polymerase (i.e., assisting the RNA polymerase to structure the DNA), curved DNA enhances the transition from heparin sensitive closed complex (RP$_C$) to a heparin-resistant, closed complex (RP$_C$), which is easily isomerized to form the open promoter complex. This allows more efficient transcription initiation when this step is rate limiting. Since the DNA in RP$_C$ is believed to be partially untwisted, curved DNA appears to aid DNA untwisting by RNA polymerase. The nature of the interaction between E. coli RNA polymerase and DNA upstream of the -35 region. The interaction between RNA polymerase and DNA upstream of the
curved DNA would be sequence independent if salt can be used to disrupt these interactions. In other words, addition of salts such as NaCl interfere with the electrostatic interactions between RNA polymerase and the phosphate charges on the DNA. As the salt concentration increased to 150 or 200 mM, wrapping diminished for both Alu156 and Ball29 promoters. The DNase I pattern consistent with RNA polymerase bound to the promoter was observed at every salt concentration. This indicates that the population of DNA-RNA polymerase complexes formed at low salt concentration were different to those formed at high salt concentration. The wrapped DNA complex was formed at low salt concentration suggesting that electrostatic interactions predominant.

Existence of unwrapped open promoter complexes. Since presence of different DNA-RNA polymerase complexes were shown in various salt concentrations, it was important to show if any of these complexes represent open promoter complexes. Potassium permanganate was used to test for open promoter complex formation as a function of salt concentration. While open promoter complexes were formed at all salt concentrations tested, open promoter complex formation decreased with increasing the NaCl concentration. Considering that no wrapped DNA complexes were observed above 150 mM NaCl there were open promoter complexes in which the DNA is no longer wrapped. In order for RNA polymerase to engage in active transcription wrapping must be released at some point.

Presence of unwrapped open promoter complexes are in agreement with this proposal. Consistent with the proposed model, this indicates that wrapping must occur in steps prior to open promoter complex formation.
DNA wrapping in the absence of open promoter complex formation. According to our model, for DNA wrapping to aid strand separation, wrapping must exist prior to formation of the open promoter complex in which the strands are separated. The step of strand separation is rate limiting at low temperatures. Thus the effect of temperature on the structured complex of Alu156 was studied. At 0°C, as expected, almost no open promoter complexes were found. Open promoter complexes were observed at 5°C, and the formation of these complexes increased when temperature was raised. Although minor differences were observed in the DNase I digestion patterns upstream of the curved DNA at each temperature, DNA wrapping was obvious at all temperatures. The minor changes in the footprint indicates that there were different complexes formed at various temperatures. The evidence supports the presence of DNA wrapping in the absence of open promoter complex formation. This suggests that wrapping can occur in complexes preceding the open promoter complex.

In our study, we went one step further and confirmed the presence of closed, wrapped promoter complexes. This would be expected if indeed wrapping occurs prior to strand separation. Many closed complexes are sensitive to effect of heparin. Heparin challenge was used for complexes formed at 37°C and 0°C. Results indicate that at 37°C all wrapped complexes were eliminated upon addition of heparin. Heparin challenge at 0°C confirmed the idea that wrapped complexes were heparin sensitive, closed complexes. Most of the complexes formed at 0°C dissociated in the presence of heparin including all the wrapped complexes. Since few open complexes were observed at 0°C, all wrapped
complexes were closed, and heparin sensitive. This agrees well with the model that the wrapped complex is the RPc1 complex.

Comparison of the effect of AT-rich regions and curved DNA on wrapping and transcription initiation. It has been established that the α subunit of E. coli RNA polymerase binds a specific AT-rich region upstream of the -35 region. It has been suggested that A-tract curvature simply functions as an α binding site (Ross et al., 1993). A collection of promoters containing AT-rich region upstream of promoter were studied and compared to Alu156 promoter. Based on the results of DNase I footprints from these promoters, wrapping was more evident for wild-type Alu156. However, small degree of wrapping was observed for AluUnc and AluUp promoters. This supports the idea that the curved DNA may stabilize a transient complex, formed by RNA polymerase with all promoters, long enough to be detected by DNase I footprinting. The results from gel retardation assay suggested that binding of E. coli RNA polymerase was strongest with the promoter with curved DNA. This suggests that there are more tight binding complexes in the population of RNA polymerase-promoter complexes formed in the presence of curved DNA. Some researchers have suggested that A-tract curved DNA is simply an AT-rich binding site for α subunit of RNA polymerase. Although we agree with part of that suggestion that curved DNA could act as an α binding site, our study showed that none of the AT-rich DNA containing promoters, even the AluUp promoter, which contain the so called UP element, were able to perform as well as Alu156. In addition, we showed that not all AT-rich regions are beneficial. In our Alu27AT promoter, the long stretch of AT
seemed to be detrimental to promoter function. Perhaps for an AT-rich region to be good α binding site, proper alignment of the α binding site relative to the promoter is necessary.

**Contribution of α subunit of RNA polymerase on wrapping.** The *E. coli* RNA polymerase containing C-terminal truncation of 94 amino acids is stable *in vivo* and assembles into holoenzyme. In other studies, this enzyme failed to protect the UP element DNA of ribosomal RNA promoter *rnbP1*. In other words, mutant α subunit could not bind to its AT-rich binding site (Ross et al., 1993). We used this enzyme to explore the effect of α subunit on DNA wrapping. DNase I footprints of this enzyme to the promoter region was the same as that of wild-type enzyme. However, DNA wrapping for promoters containing curved DNA was diminished when the mutant enzyme was used. This indicates that A tracts involved in DNA curvature may function as α subunit binding site and contribute to DNA wrapping.

**Summary of the model.** Based on the present study, we concluded that the C-terminal domain of α contacts the A-tract curvature and promoters DNA structuring by the RNA polymerase. Wrapping appears to be limited to RP<sub>c1</sub>, a heparin sensitive, closed complex. The curved DNA promotes the transition from RP<sub>c1</sub> to RP<sub>c2</sub>, a heparin resistant, closed complex. The RP<sub>c2</sub> can quickly isomerize to RP<sub>o</sub>, an open promoter complex lacking DNA wrapping. The effect of DNA curvature on overall transcription will only be observed under conditions in which the RP<sub>c1</sub> to RP<sub>c2</sub> transition is the rate-limiting reaction.
REFERENCES


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VITA

Parisa Jazbi received her bachelor of science degree in Zoology from Shiraz University in Shiraz, Iran. In August 1993, she joined the graduate program in the Department of Microbiology at Louisiana State University. She has worked as a teaching assistant for past four years. Her research focuses on investigating the role of DNA wrapping in initiation of transcription using E. coli RNA polymerase. Parisa is currently attending Louisiana State University, where she is a candidate for the doctor of philosophy degree in Microbiology with a minor in Biochemistry.
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Major Field:  Microbiology

Title of Dissertation:  DNA Wrapping and the Nature of Interaction between E.coli RNA Polymerase and Promoter DNA

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