Rotational Orientation of Upstream Curved DNA Affects Promoter Function in Bacillus subtilis*

Carl F. McAllister† and Eric C. Achberger
From the Department of Microbiology, Louisiana State University, Baton Rouge, Louisiana 70803

The Alu156 promoter isolated from the Bacillus subtilis bacteriophage SP82 is dependent on curved DNA upstream of the −35 region for efficient function. Short DNA insertions of 6–29 base pairs were used to simultaneously change the linear placement and rotational orientation of this curved DNA relative to the −35 region. When these mutant promoters were analyzed in vivo using transcriptional fusions with a chloramphenicol acetyltransferase gene, changes in rotational orientation of the curved DNA correlated with changes in promoter function. The most efficient mutant promoters contained insertions of 11 and 21 base pairs, and insertions of 15 and 25 base pairs resulted in the least efficient mutant promoters. The importance of the proper rotational alignment of the curved DNA to promoter activity was also observed in vitro at the level of transcription of RNA polymerase binding. Based on the electrophoretic mobilities of DNA fragments containing the various insertion mutant promoters, there was a second region of curved DNA downstream of the insertion point. The findings are consistent with the idea that the curved DNA deflects the helix back toward the promoter-bound RNA polymerase molecule to allow the enzyme to interact directly with upstream DNA. These interactions are proposed to structure the DNA for the formation of the open promoter complex.

The existence of static curves in DNA is well documented (1–4). In a number of cases, curved DNA is characterized by runs of adenines with a periodicity of approximately 10–11 bp.1 The degree of curvature of these regions has been estimated based on altered electrophoretic mobility of DNA fragments (1–3) and intramolecular ligation rates (5, 6). Sequence-dependent curvature has been observed in regions of DNA involved in site-specific recombination (7, 8), the initiation of DNA replication (6, 9–11), and transcription initiation.

DNA curvature and protein-mediated DNA bending are required elements for efficient transcription of some promoters. DNA bending has been implicated in transcription activation of the lactose operon promoter P1 by the cAMP-CAMP receptor protein complex (12). DNA looping has been proposed as part of the activation process of the Klebsiella pneumoniae nifH promoter by the nifA gene product (13) and the Escherichia coli gltA promoter by the gltA gene product (14). Curved DNA has been identified upstream of the E. coli ompF promoter in a region that overlaps the binding site of the transcription activator OmpR (15). DNA curvature appears to stimulate transcription in the absence of ancillary proteins. Mutation of the curved DNA upstream from promoters for a number of tRNA and rRNA operons from Gram-negative bacteria (15–19) and the bacteriophage λ P2 promoter (20, 21) reduced transcription. In addition, nucleotide sequence analysis suggests a correlation between promoters displaying high rates of transcription initiation and those with specific distributions of nucleotides associated with DNA curvature (22).

Runs of adenes upstream from promoters of Gram-positive bacteria, such as Bacillus subtilis, are a common feature (23, 24). Using a deletion analysis, it was shown that the A+T-rich DNA upstream of the spoVG promoter was required for efficient transcription by the σ24 and σ37 forms of the B. subtilis RNA polymerase (25). Curved DNA upstream of two B. subtilis bacteriophage SP82 promoters enhanced binding of the major RNA polymerase (i.e., the σ24-containing enzyme) and stimulated in vivo and in vitro transcription (26). In particular, one of these promoters, the Alu156 promoter, was strongly dependent on the curved DNA for efficient function. This promoter was selected for further study.

While it has been established that curved DNA can stimulate RNA polymerase binding to promoters, the nature of these interactions is unresolved. This report described the correlation between the precise orientation of the curved DNA relative to the promoter proper and the ability of this DNA to function as a cis-acting element for enhanced transcription from the Alu156 promoter. This structure/function relationship was investigated using mutant promoters created by the insertion of oligonucleotides between the polyadenine-induced curve and the −35 region of the promoter. Each promoter was analyzed by measuring in vivo and in vitro transcription and RNA polymerase holoenzyme binding.

EXPERIMENTAL PROCEDURES

The Alu156 promoter possesses polyadenine-induced DNA curvature upstream of the −35 region (Fig. 1). Previous studies support the idea that the curved DNA is required for efficient utilization of this promoter in B. subtilis (26). To

* Portions of this paper (including "Experimental Procedures," Fig. 6, and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass.

† The abbreviations used are: bp, base pairs; TBE, Tris-borate-EDTA.

‡ The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Portions of this paper (including "Experimental Procedures," Fig. 6, and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

1 The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J03904.
characterize the stimulation of transcription by curved DNA, a series of mutant promoters was constructed by the insertion of synthetic, double-stranded oligonucleotides of various lengths between the −35 region and the curved upstream DNA of the Alu156 promoter. These insertions served a dual purpose. First, they increased the distance between the curved DNA and the promoter by discrete increments, and second, they rotated the curved DNA around the helix with respect to the −35 region. The relevant sequence of these mutant promoters is presented in Fig. 2. For each 10.5 bp of inserted DNA, there would be a linear displacement of 3.4 nm and a rotational displacement of 360° (i.e. one full helical turn).

**Electrophoretic Mobilities of Promoter-containing DNA Fragments**—It has previously been shown that DNA fragments containing the Alu156 promoter migrated in 8% polyacrylamide gels at a rate consistent with a molecule 22% larger than its actual size (26). Deletion or replacement of the upstream DNA significantly reduced but did not entirely eliminate this aberrant mobility. This suggests that in addition to the upstream regions, the sequence downstream from the HindII cut site at −33 also exhibited some curvature. Thus, it was hypothesized that rotation of two curves relative to one another would result in a detectable change in the structure of the promoter DNA fragment. To test for altered DNA conformations, the promoters constructed in this study were excised from the vector with EcoRI and PstI and electrophoresed on 10% polyacrylamide gels at three different temperatures. It has been reported that the electrophoretic mobility of curved DNA returns to normal at elevated temperatures (19, 40). In agreement with this observation, at 65°C, DNA fragments containing the various promoters migrated at rates that were most consistent with those predicted from the nucleotide sequence derived lengths (Fig. 3). The electrophoretic mobilities of these DNA fragments were most severely retarded at 5°C and 23°C. The finding that electrophoretic mobilities were not completely restored to normal even at 65°C is consistent with the existence of a highly stable, curved DNA molecule.

Interestingly, the ratio of apparent/actual length of DNA fragments does not remain constant, as would be predicted for linear molecules of increasing length. The cyclical pattern observed in Fig. 3 would be expected if there is a region of curved DNA on both sides of the rotation point. Decreasing end-to-end distance directly correlates with decreasing electrophoretic migration rates for DNA fragments in polyacrylamide gels (41). When two regions of curved DNA are present on a single molecule, the electrophoretic mobility would be retarded most when the two curves are in the same orientation (i.e. a U-shaped DNA molecule). The electrophoretic mobility would be closest to normal when the two curves are directionally opposed forming an S-shaped molecule (41). The observed cyclical pattern had a periodicity of approximately one helical turn; the ratio of apparent/actual DNA fragment length determined at 23°C reached a maximum for the +6 and +17 mutant promoter DNA fragments and a minimum for the +15 and +25 mutant promoters. The +6 and +17 fragments should have the polyadenine regions on the opposite side of the helix relative to the wild type and +11 and +21 mutants.

If there were a single DNA curve on either side of the point of rotation, one would expect to see the points for each curve in Fig. 3 arranged so that a single sine curve be drawn through them. The data, however, show a shoulder on the right side of each peak which is most pronounced at 5°C. The most likely explanation for these patterns is the presence of a third region of curvature whose stability is enhanced at 5°C.

**In Vivo Expression from Alu156 Promoter Constructions**—Transcriptional fusions were established between each of the

---

**Fig. 1. Nucleotide sequence of the Alu156 promoter.** Bases representing the +1 transcription start site, the −35 region, and the −10 region are in **bold type**. The runs of adenines in the nucleotide sequence immediately upstream of the −35 region are in **bold type**.

---

**Fig. 2. Nucleotide sequence of DNA immediately upstream from the −35 region of the Alu156-derived mutant promoters.** The numbering of the nucleotide sequence corresponds to the right column. The oligonucleotide insertions for each mutant are underlined, and the runs of adenines are in **bold type**.

---

**Fig. 3. Effect of temperature on the electrophoretic mobility of DNA fragments containing the Alu156 promoter or insertion mutant promoters.** The ratio of the apparent length based on electrophoretic mobility to actual length determined from the nucleotide sequence is presented as a function of the length of the oligonucleotide insertion for the wild-type Alu156 and insertion mutant promoters. The apparent length of promoter-containing DNA fragments was determined using pBR322-MspI and pBR322-HaeIII size standards as described under “Experimental Procedures.” The temperatures used during electrophoresis were 5°C (●), 23°C (○), and 65°C (△).
promoters and the chloramphenicol acetyltransferase gene carried on the promoter cloning vector pPL703. The levels of chloramphenicol acetyltransferase specific activity measured for each promoter construction in *B. subtilis* are presented in Fig. 4A. From the data in Fig. 4A, it was apparent that efficient transcription from the Alu156 promoter was dependent, in part, on the rotational orientation of the upstream DNA relative to the promoter proper. The mutant constructions which allowed the highest levels of chloramphenicol acetyltransferase expression were the promoters in which the upstream DNA was rotated by one or two turns of the helix (e.g. +11 and +21 mutant promoters) and was, thus, in the same rotational orientation as the wild-type Alu156 promoter. Chloramphenicol acetyltransferase specific activities decreased to a minimum at or very near the point at which the polyadenine regions would be on the opposite side of the DNA helix from the −35 region (i.e. the +15 and +25 insertions). The high chloramphenicol acetyltransferase specific activity observed from the +6 and +9 mutant promoters may reflect a rotation-independent effect based on the proximity of the polyadenylome region to the promoter proper.

**In Vitro Binding of RNA Polymerase to Insertion Mutant Promoters**—To ascertain if the changes in *in vivo* promoter function reflected differences in the affinity of RNA polymerase for the various promoter constructions, the binding of purified *B. subtilis* RNA polymerase (core-σ70) to the wild-type and mutant promoters was examined. The wild-type promoter was allowed to compete with various combinations of three to four mutant promoters for subsaturating levels of RNA polymerase. Equal molar amounts of each promoter were used in these nitrocellulose filter binding assays. RNA polymerase binding data presented in Fig. 4B represent results from a single enzyme concentration in the competition binding assay. These data have been normalized to the level of RNA polymerase binding observed with the Alu156 promoter-containing fragment which was present in each assay. An example of the competition binding assay is presented in Fig. 5. Low recovery of the weakly bound promoters was observed until the promoters with a high affinity for RNA polymerase were bound at near-maximum level. The normalized data presented in Fig. 4B were generated at an enzyme/DNA weight ratio of 2.5 (e.g. equivalent to the 15-μg point in Fig. 5).

The binding data agree well with the results obtained from the *in vivo* expression assays. Maximum binding of RNA polymerase occurred with the wild-type Alu156 promoter and the +11 and +21 mutants which retained the original rotational orientation of the upstream DNA relative to the −35 region. Insertions that altered this orientation reduced the affinity of RNA polymerase for the mutant promoters. In addition, a mutant promoter that lacked the upstream DNA, the Alu156 extended promoter (26), was included in the competition binding assay. For this mutant, the fraction of input DNA bound was 0.12 (normalized as in Fig. 4B) at an enzyme/DNA ratio of 2.5. This level of binding was approximately one-half that observed for the least efficient insertion
mutant promoters, the +6 and +15 mutants. This suggests that while the rotational orientation of upstream and downstream curved DNA was very important in producing a DNA conformation conducive to efficient RNA polymerase binding, the presence of the curved DNA in any orientation affects binding.

There were two apparent discrepancies between the in vivo data and the in vitro RNA polymerase binding data. First, the +6 mutant appeared to bind RNA polymerase weakly. If the polyadenine region can affect promoter function by its proximity to the promoter, this effect was not evident at the level of RNA polymerase binding to a linear DNA fragment. Second, the +13 mutant promoted very low chloramphenicol acetyltransferase expression in vivo but appeared to bind RNA polymerase tightly. In separate experiments, 400 μM ATP and CTP were included in the competition binding reactions to permit transcription initiation. Just prior to filtration, these reactions were diluted in reaction buffer containing 1 M NaCl at 0 °C to dissociate non-initiated complexes (42). Filter retention of DNA fragments containing the +13 mutant relative to other promoters in the reaction was unchanged from that presented in Fig. 4B (data not shown). This suggests that RNA polymerase was able to form initiatable, open promoter complexes at the +13 mutant promoter sequence as efficiently as it could to the other promoters in this study.

Effects of Insertion Mutagenesis of the Alu156 Promoter on In Vitro Transcription—The ability of the wild-type and mutant promoters to direct transcription from linear and naturally supercoiled pUC8-derivative templates was tested using the same enzyme/DNA weight ratios as in the RNA polymerase binding experiments. In general, the data from in vitro transcription studies agreed with the in vitro binding and in vivo chloramphenicol acetyltransferase expression findings. Promoters which gave the most efficient RNA synthesis (e.g. the Alu156 promoter and the +11 and +21 mutants) also bound RNA polymerase more tightly and permitted higher levels of in vivo chloramphenicol acetyltransferase expression. Again, the promoters which gave the lowest transcription rates in vitro were those in which the polyadenine regions had been rotated to the opposite side of the helix as the −35 region.

The levels of transcription from linear versus supercoiled templates correlated to the lengths of the respective transcripts. The RNA synthesized from the linear transcripts was approximately 900 bases long, whereas transcription from the supercoiled template would be expected to generate a transcript of at least 1600 bases in length if terminated efficiently at the Ap^5 gene terminators (43). The increased incorporation of UMP seen with the supercoiled templates is fairly consistent with the series of insertions when compared to the levels from linear templates for each construction. The notable exceptions are the +15 and +25 insertion mutants. The partial unwinding of the negatively supercoiled DNA template seems to stimulate transcription from these mutant promoters. The linear displacement of the curved DNA from the promoter may contribute to this effect because transcription was not preferentially elevated on supercoiled DNA templates for the +6 mutant and the effect was greater for the +25 mutant than for the +15 mutant (Fig. 4C).

DISCUSSION

The orientation of the curved DNA immediately upstream of the −35 region was altered by the introduction of short oligonucleotides between the curved DNA and the rest of the promoter. The insertion of these oligonucleotides resulted in both linear and rotational displacement of the upstream DNA in relation to the promoter. In general, insertion mutant promoters in which the curved DNA was rotated away from the original orientation displayed reduced function. Insertions of 11 or 21 bp that maintained the original rotational orientation while linearly displacing the curved DNA produced mutant promoters that retained most of the activity of the wild-type promoter. These results suggest that a proper rotational orientation is the most critical factor for the stimulation of transcription by the upstream curved DNA.

There was a minor component of transcription enhancement based on the proximity on the curve to the promoter. The insertion mutant promoter that displayed the lowest activity (i.e. chloramphenicol acetyltransferase specific activity, RNA polymerase binding, and in vitro transcription) retained more of the original promoter function than mutant promoters in which the upstream curved DNA was deleted (26). This indicates that the curved DNA could stimulate transcription (e.g. approximately 2-fold) even when the rotational orientation was not optimal. This may explain, in part, why the +6 and +9 insertion mutants exhibited efficient promoter function in vivo.

The proximity of the curved DNA to the promoter may also govern the effect of DNA supercoiling on promoter function. DNA supercoiling has been shown to stimulate transcription from Alu156 mutant promoters lacking the curved upstream DNA (26). For the most part, this effect was not observed for insertion mutant promoters. Slightly elevated transcription was observed on supercoiled templates containing the +15 mutant; this effect was more pronounced for the +25 mutation. While curved DNA has been shown to influence the topology of supercoiled molecules (44), it is not known how this may affect protein-DNA interactions.

Polyacrylamide gel electrophoresis of DNA fragments containing the various Alu156 promoter constructions demonstrated the presence of a second region of curved DNA in addition to the polyadenine-dependent curvature just upstream of the −35 region. The cyclical pattern of the curve generated in a plot of apparent fragment length/actual length versus insert length (Fig. 3) indicated the presence of a second curve downstream of the point of rotation (i.e. the site of the insertion). This second curve may be induced by the pairs of adenes at −5 and +5 (Fig. 1). Adenine dinucleotides associated with the −10 region have been proposed as a possible site of DNA curvature (4, 45). Also, the asymmetry of the cyclical pattern presented (Fig. 3) suggests the presence of a third region of altered DNA conformation. The DNA upstream of −90 is characterized by the presence of runs of 2–4 adenes and thymines. The length and periodicity along the helix of these runs of nucleotides marginally conforms to the sequence motif associated with curved DNA (3, 5). It would not be unexpected that the DNA curvature induced by this sequence would be stabilized at low temperature as depicted in Fig. 3. Although it is not known if DNA upstream of −80 is important for transcription from the Alu156 promoter, replacement of DNA upstream of −33 with the analogous DNA from the Ball29 promoter resulted in the loss of only 35% of the function in vivo (26). This curved upstream DNA from the Ball29 promoter terminates at −77 and lacks DNA for additional upstream curves.

Several models have been suggested to explain the role of curved DNA in the activation of transcription. It has been proposed that the curved upstream DNA acts as a sequence-independent RNA polymerase binding site to maintain a locally high concentration of enzyme and, hence, facilitate binding to the promoter (22, 46). This model cannot adequately account for the effect of curved DNA on the stability
of RNA polymerase-promoter complexes once formed (26) or the effect of altered rotational orientation on RNA polymerase binding described in this work.

The overall shape of the promoter DNA may influence the efficiency of transcription initiation. Based on electrophoretic mobility studies, DNA containing the wild-type Alu156 promoter is "S"-shaped. It has been proposed (49) that promoter DNA is structured by wrapping it around the RNA polymerase prior to helix untwisting and DNA strand separation. The presence of the two curves in the S-shaped promoter DNA may facilitate DNA wrapping and allow the RNA polymerase to stably contact both sides of the DNA helix.

The positioning of the runs of adenes and thymines immediately upstream of the Alu156 promoter would deflect the DNA toward the side of the helix to which the majority of the interactions between the major B. subtilis RNA polymerase and other promoters have been mapped (47, 48). This suggests that the enhanced promoter function is dependent on the interaction between the RNA polymerase and the upstream DNA either directly (19, 22) or through a second RNA polymerase bond to the region (49). In each case, the orientation of the curve would be predicted to be critical. Several lines of evidence are consistent with the involvement of a second RNA polymerase molecule in the efficient transcription of the Alu156 promoter. Although nucleotide sequence analysis identified a site with fair homology with the consensus promoter sequence located upstream of the promoter, most of the promoter function was retained (26). The positioning of the runs of adenines and thymines immediately upstream of the productive -35 region and the curved DNA did not appreciably influence the interactions between the major -35 region and the curved DNA, which has just this region (data not shown). When the upstream DNA of the Alu156 promoter was replaced with the analogous DNA from the Ball29 promoter, most of the promoter function was retained (26). The upstream DNA of the Ball29 promoter exhibited DNA curvature but has no secondary binding site based on nucleotide sequence analysis or RNA polymerase binding measured by the nitrocellulose filter assay. In addition, the shape of the curve in Fig. 5 does not support a model predicting the cooperative binding of more than one enzyme molecule. In other studies, chemical and enzymatic probes were used to demonstrate a secondary RNA polymerase binding site immediately upstream of the productive B. subtilis veg promoter (48). Although transcription from the promoter was slightly increased in the presence of the secondary binding site, effective transcription was still observed when the second site was deleted (50).

If the RNA polymerase molecule bound to the promoter makes direct contact with the upstream curved DNA, one would expect that not only the rotational orientation but also the linear placement of the curved DNA to be critical to RNA polymerase binding. The insertion of 21 bp of DNA between the -35 region and the curved DNA did not appreciably disrupt RNA polymerase binding. This suggests that the curve may loop the DNA and permit the RNA polymerase to also bind DNA upstream of the curve. Linear displacement of the curve would be less of a factor if the function of the curve is to mediate the looping of the DNA back around the RNA polymerase. If the DNA curve facilitates DNA looping upstream of the Alu156 promoter, the DNA loop must be relatively small. The Alu156 hybrid promoter (26), which has just 54 bp of DNA upstream of the -35 region, was bound by RNA polymerase nearly as efficiently as the wild-type Alu156 promoter.

Acknowledgments—We thank Drs. H. D. Braymer and M. R. Belas for critical reading of the manuscript.

References

43. von Gabain, A., Belasco, J. G., Schettel, J. L., Chang, A. C. Y.,
Positioning of Upstream DNA and Promoter Selection


Materials
Restriction enzymes and DNA modifying enzymes were purchased from Bethesda Research Laboratories or New England Biolabs, Inc. Dideoxynucleotides were obtained from Pharmacia, Inc. (5′)- or (3′)-dideoxynucleotides and [α-32P]dATP were used at 2 to 3× 106 cpm/nmol. Restriction enzymes were purchased from New England Nuclear. Nucleotides were prepared from human erythrocyte by the procedure of Hill et al. (1980) Nucleic Acids Res. 8, 2221-2235.

Synthesis and purification of oligonucleotides: The oligonucleotides were synthesized by the phosphoramidite method using an Applied Biosystems DNA synthesizer model 380B. Purification of the oligonucleotides was either by the procedure of Brown and Mager (1980) J. Mol. Biol. 135, 103-125 or by ethanol precipitation followed by the procedure recommended by the manufacturers. The oligonucleotides were stored in aqueous solution at 4°C. They were reconstituted in 0.5 M NaCl, 10 mM Tris, 1 mM EDTA to a 1 mg/ml concentration.

Synthesis of DNA: A 50 ng gel retardation assay for specific DNA fragments was used to isolate the target DNA fragment. DNA fragments that were used for the reaction were digested with restriction enzymes and purified by electrophoresis on a 1% agarose gel. The DNA fragments were then labeled with [32P]dATP by the procedure of Ausubel et al. (1987) Methods Enzymol. 164, 208-217. The DNA fragments were separated by electrophoresis in 1× TBE buffers at 4°C. The DNA fragments were purified by ethanol precipitation and redissolved in 0.5 M NaCl, 10 mM Tris, 1 mM EDTA.

The DNA fragments were then used to isolate the target DNA fragment. The DNA fragments were then labeled with [32P]dATP by the procedure of Ausubel et al. (1987) Methods Enzymol. 164, 208-217. The DNA fragments were separated by electrophoresis in 1× TBE buffers at 4°C. The DNA fragments were purified by ethanol precipitation and redissolved in 0.5 M NaCl, 10 mM Tris, 1 mM EDTA.

The DNA fragments were then used to isolate the target DNA fragment. The DNA fragments were then labeled with [32P]dATP by the procedure of Ausubel et al. (1987) Methods Enzymol. 164, 208-217. The DNA fragments were separated by electrophoresis in 1× TBE buffers at 4°C. The DNA fragments were purified by ethanol precipitation and redissolved in 0.5 M NaCl, 10 mM Tris, 1 mM EDTA.

The DNA fragments were then used to isolate the target DNA fragment. The DNA fragments were then labeled with [32P]dATP by the procedure of Ausubel et al. (1987) Methods Enzymol. 164, 208-217. The DNA fragments were separated by electrophoresis in 1× TBE buffers at 4°C. The DNA fragments were purified by ethanol precipitation and redissolved in 0.5 M NaCl, 10 mM Tris, 1 mM EDTA.