Evidence for DNA Bending at the T7 RNA Polymerase Promoter

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Phage T7 RNA polymerase is the only DNA-dependent RNA polymerase for which we have a high-resolution structure of the promoter-bound complex. Recent studies with the more complex RNA polymerases have suggested a role for DNA wrapping in the initiation of transcription. Here, circular permutation gel retardation assays provide evidence that the polymerase does indeed bend its promoter DNA. A complementary set of experiments employing differential phasing from an array of phased A-tracts provides further evidence for both intrinsic and polymerase-induced bends in the T7 RNA polymerase promoter DNA. The bend in the complex is predicted to be about 40-60° and to be centered around positions -2 to +1, at the start site for transcription, while the intrinsic bend is much smaller (about 10°). These results, viewed in the light of a recent crystal structure for the complex, suggest a mechanism by which binding leads directly to bending. Bending at the start site would then facilitate the melting necessary to initiate transcription.

Introduction

DNA conformational changes have been demonstrated to play an important role in many biological processes. The processes of DNA packaging into histones or transcription activation, for example, can involve protein-DNA interactions via DNA bending. Many regulators of transcription bend their DNA target to optimally position the DNA, or proteins bound to the DNA, with respect to the RNA polymerase (Perez-Martin & Espinosa, 1994). DNA bending has also been observed for the Escherichia coli and mitochondrial RNA polymerases bound to their respective promoters in the absence of regulatory proteins (Craig et al., 1995; Kuhnke et al., 1989; Meyer-Almes et al., 1994; Rees et al., 1989; Schinkel et al., 1988). For the E. coli RNA polymerase, evidence suggests an approximately 45° bend centered near the transcription start site, and perhaps even more extensive DNA wrapping around the polymerase upstream of the start site (Craig et al., 1995; Darst et al., 1989; Kuhnke et al., 1989).

Phage T7 RNA polymerase is a single subunit enzyme, which carries out all the fundamental steps of transcription without the help of activator proteins. The availability of a published high-resolution crystal structures with and without promoter DNA makes T7 RNA polymerase an ideal model system in which to study DNA conformational changes and structure-function relationships during the binding of RNA polymerase to its promoter.

The crystal structure of T7 RNA polymerase (Cheetham et al., 1999; Jeruzalmi & Steitz, 1998; Sousa et al., 1993) shows that the protein is organized around a DNA binding cleft with fingers, palm and thumb sub-domains similar to other DNA and RNA-dependent polymerases (Joyce & Steitz, 1994; Sousa, 1996; Sousa et al., 1993). The recent structure of the enzyme bound to promoter DNA confirms that the enzyme recognizes promoter contacts upstream of position -5 via direct read out of major and minor groove contacts in a segment of duplex which is close to linear (Cheetham et al., 1999). This structure, which was formed with an oligonucleotide duplex extending only to position -1, clearly shows an open complex, the enzyme interacting with the template strand near the start site for transcription. It must be noted, however, that because of the DNA construct used, the crystal structure cannot comment on the nature of the DNA downstream of the promoter. In fact, recent studies have challenged the notion that the statically bound complex is predominantly open in a (more normal) complex formed with DNA...
extending downstream of the start site (Villemain et al., 1997).

The crystal structure similarly provides no information on the nature of the DNA upstream of the promoter (upstream of position –17). Various models for promoter binding in more complex RNA polymerases have implicated a role for DNA wrapping (around the polymerase) in the initiation of transcription (Coulombe & Burton, 1999; de Haseth & Helmann, 1995; Robert et al., 1998). The T7 RNA polymerase consensus promoter is A + T-rich at its upstream end, and many phage promoters are A + T-rich extending upstream of position –17, suggesting a possible role for bending immediately upstream of the promoter.

Here, we have used gel electrophoretic mobility shift methods to present evidence not only for a 40°-60° polymerase-induced bend in the DNA, but also for a smaller 9° intrinsic bend in the absence of polymerase. Contrary to simple expectations from some of the above models, the data suggest that the polymerase-induced bend is not localized upstream of the promoter, but is instead located closer to the start site. To probe these possibilities, mutations were introduced into the promoter to look for sequence effects on bending.

Results

Here, we have used two electrophoretic assays to provide evidence for bending of the T7 RNA polymerase promoter induced by the statically bound RNA polymerase. The circular permutation assay relies on position-dependent variations in electrophoretic mobility of protein-DNA complexes. Bends located in the middle of a fragment perturb the observed mobility more than bends placed at the ends of identically sized fragments. The phasing assay places the target binding site at varying distances from an intrinsic bend in the DNA. A bend in the target can either add to or subtract from the effects of the intrinsic bend. Position or phase-dependent variations in mobility are most readily interpreted in terms of a protein-induced bend at the target site.

Circular permutation assay

The circular permutation assay was used initially to determine whether T7 RNA polymerase bends its promoter, as has been demonstrated for the E. coli and mitochondrial RNA polymerases (Kuhnke et al., 1989; Schinkel et al., 1988). An oligonucleotide representing the consensus promoter sequence (construct 1 in Figure 1) was cloned into the permutation vector pSL6 (Gaal et al., 1994). DNA probes of the same length, with the promoter at different positions, were generated as described in Materials and Methods. During the PCR amplification, the DNA was uniformly labeled via the incorporation of [α-32P]dATP. The PCR amplified and Qiagen column-purified DNA was then digested with the indicated restriction enzyme, desalted on a Qiagen column, and used directly. Consequently, the circular permutation gels also show shorter DNA fragments arising from the restriction enzyme-cleaved ends, which do not carry the promoter. Initially, digests with only four restriction endonucleases were analyzed. Two of the resulting fragments place the promoter near the center of the DNA, while two place the promoter at opposite ends of the fragment.

The results shown in Figure 2 reveal that free promoter-containing DNA does not display detectable mobility differences in this assay (but see below). In contrast, polymerase-promoter complexes carrying the promoter at the ends migrate faster than ones with the promoter at the center. This position-sensitive variability in mobility is generally interpreted to arise from increased DNA bending or flexibility (Wu & Crothers, 1984; Zinkel & Crothers, 1990). Kerppola & Curran (1991) have

![Figure 1](image)

**Figure 1.** Promoter constructs used in the bending experiments. Construct 1 contains the conserved T7 promoter sequence from position –17 to position –1 and the semi-conserved A + T-rich sequence from position –21 to position –18. The sequences of the second, third and fourth promoters have been modified to study the effect of specific substitutions on bending. Modifications are indicated in bold letters.
pointed out that structures other than a static DNA bend may lead to similar alterations in the electrophoretic mobilities, and therefore propose the use of the term DNA flexure (intended to remain ambiguous) to refer to the source of the anomalous gel mobilities.

Figure 2. (a) and (b) Circular permutation assay with the native promoter (construct 1) and (c) and (d) with the promoter that is G+C-rich upstream of position -17 (construct 2). (a), (c) Comparison of the mobilities of polymerase-promoter complexes to mobilities of A-tract standards (2A through 5A, containing two to five A-tract repeats) with different bending angles. For the DNA constructs containing the T7 RNA polymerase promoter, 163 bp long DNA fragments were generated by restriction digestion with different endonucleases, as described in Materials and Methods. The distances (in base-pairs) of position -6 from the end of the DNA are indicated on the top of the lanes. This distance is indicated by a broken line in the cartoon at the top of the Figure, with the promoter indicated by an open rectangle, and the direction of transcription from position -1 shown by an arrow. For the polymerase complexes, approximately 5 nM DNA was incubated with 75 nM T7 RNA polymerase, in the presence of 2 μg/ml salmon sperm DNA (final concentrations) to prevent non-specific binding. The letters f and c indicate the positions of the free DNA and the T7 RNA polymerase-promoter complex, respectively. (b), (d) Relative mobilities of free (open circles) and bound DNA (filled circles) were plotted versus the distance in base-pairs of position -6 from the end of the DNA fragment. Data for the complex were fit to equation (2). The location of the bend in the bound complex was estimated to be position +1 (distance = 75) for both promoter constructs, and the bend angle is expected to be approximately 39° and 37° for constructs 1 and 2, respectively.
Complexes containing fragments with the promoter located symmetrically at opposite ends run differently from each other, suggesting that the DNA flexure is not located symmetrically within the promoter. To estimate the magnitude and the location of the flexure, the normalized mobilities of the T7 RNA polymerase-complexes were fit to equation (2). Here, promoter position $\beta$ was arbitrarily chosen as a reference point, and $\Delta x$ is the distance from position $-6$ to the center of the bend. Fitting the data to equations (2) and (3), the center of the bend is preliminarily estimated to lie near position $\beta-2$, and the magnitude of the bend is estimated to be $39^\circ$. For the latter analysis, running A-tract standards with known bending angles on the same gels as the polymerase-promoter complexes provided an internal calibration of the flexure angles.

The fits in Figure 2(b) and (d) have relatively few data points. To confirm the interpretation that bending localizes near the start site and not upstream of the promoter, circular permutation assays of the native promoter digested with ten different endonucleases are shown in Figure 3 (DNA standards were interspersed on the same gel to ensure that the obtained migration differences are not due to “smiling” of the gel). Fitting of this larger data set, as shown in Figure 3(b), places position $-2(\pm 3)$ as the predicted location of the flexure. This result argues strongly that the observed DNA flexure is not located in the upstream part of the promoter, but is located at the downstream end of the promoter, near the start site.

The apparent localization of bending to near the start site does not preclude additional bending of the promoter at other locations (although the determination of the bending locus would be complicated by the presence of more than one bend in the DNA). As summarized in the introduction, there was some reason to expect a bend in the region upstream of the start site. Both the consensus promoter sequence from position $-13$ to $-17$ and the less conserved region of the bacteriophage DNA upstream of position $-17$ are characteristically A+T-rich. In order to test for the possibility of bending in this region which might require the presence of an A+T-rich sequence, A-T base-pairs were replaced by G-C base-pairs upstream of position $-17$ in promoter construct 2 (retaining the full consensus promoter sequence), and upstream of position $-13$ in promoter construct 3 (altering the consensus sequence from position $-14$ to $-17$). The complex formed with the former construct shows an electrophoretic profile indistinguishable (predicting a $37^\circ$ bend) from that of the native promoter (and has wild-type activity in in vitro assays of transcription; data not shown), indicating that the A+T-rich region beyond the promoter is not required for the observed bending.

In contrast, we were not able to obtain a gel shift using the third promoter construct (G+C-rich upstream of position $-13$), presumably due to weaker polymerase binding to this sequence or to dissociation of the complex during electrophoresis. This construct also shows a tenfold reduction in the steady-state synthesis of run-off 9mer RNA (data not shown). Consequently, although this region of the DNA is essential to binding, we can reach no conclusions regarding the requirement of this region for the observed bending.
Phasing analysis

Polymerase-promoter complex

Phasing analysis is believed to be a better test of static DNA bending (Ansari et al., 1995; Kerppola & Curran, 1997; Salvo & Grindley, 1987) and so was used here as a complement to the circular permutation assay. Phasing plasmids were constructed in which the promoter sequence is separated by a variable length linker from a stretch of three phased A-tracts. An oligonucleotide carrying the consensus promoter sequence was inserted into plasmids pTK401-26 and pTK401-28 as described in the Materials and Methods and shown in Figure 4. The complex formed shows a very clear phase-dependent variation in its mobility, as shown in Figure 5(a), consistent with the above permutation results. The relative mobilities were fit to equation (4) (see Figure 5(b)) to obtain the phasing amplitude ($A_{PH}$). Fitting the data to equation (5), the angle of the polymerase-induced bend is estimated to be 63°.

The permutation analysis predicts a bend centered near the start site. To assess whether such bending requires the characteristic TATA sequence from position -4 to -1, a further construct (construct 4) was prepared in which the A:T and T:A base-pairs at positions -3 and -2 have been replaced by G:C and C:G base-pairs, respectively. These substitutions are designed to change the TATA sequence immediately upstream of the promoter start site to the sequence TGCA, which is predicted to have a 3-4 kcal/mol higher energetic barrier to melting (Breslauer et al., 1986; SantaLucia et al., 1996; Sugimoto et al., 1996). In run-off transcription of a nine base transcript, while initiation occurs with specificity for this construct, activity is down threefold (data not shown). Qualitative gel shift binding assays were carried out to ask whether the decreased transcription rate is due to weakened binding. The amounts of polymerase-promoter complexes were quantified as a function of increasing enzyme concentration and the results show that the affinity of the promoter with a TATA to TGCA substitution (construct 4) for T7 RNA polymerase appears to be on the same order of magnitude as that of the native promoter (although this assay is not well-suited for accurate measurements in the T7 system, it should be able to distinguish large decreases in binding affinity).

Phasing analysis was carried out for the promoter containing the TATA to TGCA substitution, as shown in Figure 5. Large phase-dependent mobility changes are observed, and were fit to equation (4) as described above. Using equation (5) the angle of the bend was estimated to be 60°, comparable to that for the consensus promoter.

Intrinsic bend in the promoter

Phasing analysis is more sensitive than the permutation assay, and was also able to detect intrinsic DNA bending in the absence of polymerase. Intrinsic bending angles of 9° and 10° were calculated for promoters 1 and 4, respectively. To compare intrinsic DNA bending for promoters 1, 3 and 4, phasing analyses were run on the free DNA probes as shown in Figure 6. To ensure that the salt conditions in the gel do not effect the migration pattern of the free probes, in this case TBE was used instead of the Tris-glycine gel running buffer. Bending angles of 8° and 11° were observed for the native promoter 1 and for promoter 4, respectively. These numbers are similar to the values determined previously with the Tris-glycine buffer, and indicate that the measured bending angles do not vary significantly with gel conditions. Interestingly, the promoter which is G+C-rich upstream of position -13 shows smaller amplitude variations, and although the fit was not as good as for the other constructs, the data are consistent with a bend of less than 4° for this sequence.

Comparison of the assays

Both gel mobility shift methods used in this work have advantages and disadvantages. It has been argued that the circular permutation assay may be misinterpreted if the structure of the protein itself has position-dependent effects on the migration of the complex (Ansari et al., 1995; Kerppola & Curran, 1997). Indeed, mobility differences were observed for the Hg-MerR-DNA complex in circular permutation assays, but not

![Figure 4](image)

Figure 4. Design of probes for phasing analysis. The distance of position -6 (within the promoter) from the center of the A-tract was varied from 30 to 40 base-pairs, in two base-pair increments. The sequence of the non-template strand for the inserts is indicated.
In phasing analyses, and the authors concluded that the DNA is not bent (Ansari et al., 1995; Ansari et al., 1992). The cause of mobility differences observed in phasing analysis has also been disputed for proteins with elongated structures. Although the mobility differences measured in phasing analyses argue for DNA bending for the protein-DNA complexes of the proteins Fos and Jun, no evidence for DNA curvature in this system was found in a minicircle competition binding assay (Kerppola, 1997; Sitlani & Crothers, 1998).

The shape of the T7 RNA polymerase molecule is relatively globular, so that we do not expect the position-dependent gel mobilities of the complex to be dominated by the protein structure and shape, but mostly by the conformation of the 163-360 base-pair DNA. By comparison with the A-tract standards, the DNA bending angle for the native T7 RNA polymerase promoter complex was estimated to be $39^\circ$ and $63^\circ$, from the circular permutation and phasing analyses, respectively. Since the circular permutation assay yields smaller mobility differences (15% overall) than does the more sensitive phasing assay ($\approx 70\%$), we expect that the actual value of the bend angle is closer to $60^\circ$.

**Discussion**

Two different approaches have been used to assess the characteristics of a potential bend in the static complex between T7 RNA polymerase and its promoter. The circular permutation assay is well suited to predict the location of a bend in the DNA, while the phasing analysis can provide
information on the direction of a bend. Both assays provide evidence for a polymerase-induced bend in the DNA, while the phasing analysis additionally provides evidence for a smaller intrinsic bend in the absence of RNA polymerase.

The observed mobility variations of DNA fragments containing the consensus, unbound promoter spaced at different distances from an intrinsically bent array of A-tracts provides evidence for a small, but significant intrinsic bend in the promoter. Although a permutation analysis of the unbound promoter was not possible, phasing analyses of unbound mutant promoters can help to localize the intrinsic DNA bend of the native sequence. The intrinsic DNA bending observed for the construct with a modified TATA sequence adjacent to the start site (construct 4) does not change significantly compared to that of the native sequence (10° versus 9°, respectively), suggesting that sequence-dependent, intrinsic DNA bending does not occur at positions –2 or –3. Interestingly, promoter construct 3, which replaces A·T and T·A base-pairs upstream of position –13 by G·C and C·G base-pairs, respectively, does not show a significant overall DNA bend in the phasing assay. This suggests that an intrinsic bend in the native promoter may be located upstream of position –13 and is eliminated by these G·C base-pair substitutions (or, less likely, that a newly introduced bend in this upstream region exactly cancels a downstream curvature).

Evidence for polymerase-induced bending of the promoter DNA near the start site

The circular permutation assay suggests that binding of T7 RNA polymerase to its promoter results in a bend centered near the transcription start site (constructs 1 and 2, containing the native promoter sequence between positions –1 and –17). The same permutation analysis has also placed the location of induced DNA curvature near position +1 for the E. coli enzyme (Kuhnke et al., 1989), and was explained by the formation of a transcription bubble (melting) in this region.

Several lines of evidence support melting of DNA near the start site in the T7 RNA polymerase binary complex. The non-template strand around the start site (positions –6 to –4) shows increased sensitivity to single-strand-specific endonucleases in the polymerase-promoter complex (Muller et al., 1989; Strothkamp et al., 1980). In addition, the fluorescence intensity of a 2-aminopurine probe placed at several positions in the TATA region increases upon enzyme binding, clearly indicating a conformational change in the DNA in that region (Jia et al., 1996; Ujvári & Martin, 1996). Polymerase-induced potassium permanganate hyper-reactivity also localizes within the TATA sequence and indicates enhanced access to the 5-6 double bond in the pyrimidine ring of the thymine bases, consistent with melting in this region (Place et al., 1999).

However, for both the T3 and T7 RNA polymerases and for E. coli RNA polymerase, DNase I hypersensitivity was observed upstream of the melted region of each promoter (Basu & Maitra, 1986; Craig et al., 1995; Place et al., 1999). These observations have led to proposals that upstream promoter wrapping around the enzyme may be a common mechanism for promoter binding to these polymerases (Place et al., 1999). Instead, our permutation assay places the locus of the induced-bend at the downstream end of the promoter, near the start site. Although we cannot rule out the possibility of a small additional bend upstream of position –17, a large bend upstream of the promoter seems unlikely, as this would likely move the apparent bending locus farther upstream.

Villemain et al. (1997) have recently proposed that in the absence of NTPs, the closed complex dominates over the open complex approximately 7:1, and that binding of the initiating nucleotide

Figure 6. Comparison of intrinsic bending for native and modified promoters, in the absence of RNA polymerase. (a) Phasing analyses with promoter constructs 1 (native, filled circles), 3 (G + C-rich upstream of position 13, crosses) and 4 (TATA to TGCA, open circles). (b) Normalized mobilities were plotted versus the distances (in base-pairs) of position –6 from the middle of the A-tract and were fit to the phasing function as described in the text. The intrinsic DNA bend angles are expected to be 8°, <4° and 11° for constructs 1, 3 and 4, respectively.
drives the complex towards the open form. These conclusions are derived from the fitting of steady-state kinetic data as a function of the concentration of GTP. However, product (pppGpG) re-association with the polymerase-promoter complex was not included in the equations leading to this kinetic model (Villemain et al., 1997), and the results of Jia & Patel (1997) have demonstrated that this reaction cannot be neglected. By observing the topological unwinding of the DNA in a topoisomerase assay, Villemain et al. (1997) also found that open complex formation on plasmids could be observed only in the presence of the initiating nucleotide (GTP). Basu & Maitra (1986) and Place et al. (1999) have reported their inability to footprint the polymerase-promoter complex in the absence of GTP. This most likely arises from the reasonably strong affinity of T7 RNA polymerase for non-promoter DNA, which predicts relatively low promoter occupancy in the presence of a large excess of non-promoter DNA. Indeed, in our bending assays, which place the 22 bp promoter within a 160-360 bp fragment, apparent occupancy can be as low as 5% (under conditions which would predict >90% occupancy in the absence of competition). Thus, the lack of observed topological unwinding in the absence of GTP might simply arise from low promoter occupancy (the subsequent addition of GTP would drive more stable complex formation and increase promoter occupancy).

A complex between T7 RNA polymerase and a short double-stranded DNA fragment corresponding to the T7 promoter sequence from position –17 to –1 has recently been crystallized with T7 RNA polymerase (Cheetham et al., 1999). The structure, shown in Figure 7, reveals only a very slight distortion in the upstream duplex promoter region centered near position –10. However, the strands are separated downstream of position –5, and while the non-template strand cannot be resolved downstream of this position, the template strand deviates substantially from a B-form path. This suggests that the DNA is melted in the static complex and would almost certainly lead to an overall bend in the DNA centered near the transcription start site. DNA bending by the related phage SP6 RNA polymerase has also recently been observed by electron microscopy (during the elongation phase of transcription (Bednar et al., 1999)). This observation, combined with our bending results, suggests that the polymerase bends DNA not only upon initial binding, but also throughout transcription.

Potential relationships between DNA bending, binding and open complex formation

During open complex formation, different conformational changes of the promoter have been proposed to take place in the E. coli RNA polymerase system. According to the “twisting-and-opening” model, binding of the polymerase to the –35 and –10 elements with a slight twist could unwind the intervening DNA, which would lower the energetic barrier to base-pair opening (deHaseth & Helmann, 1995). Alternatively (or in addition), DNA bending could also lower the barrier to melting of the DNA strands, as reviewed by Perez-Martin et al. (1994) and deHaseth et al. (1998). Gel shift experiments, atomic force microscopy, footprinting and chemical probing experiments all provide strong experimental evidence for polymerase-induced promoter bending in this system (Craig et al., 1995; Kuhnke et al., 1989; Rees et al., 1993). The theoretical calculations of Ramstein & Lavery (1988) have shown that DNA base-pair opening in the sequence (dA5)(dT5) is strongly related to the observed intrinsic DNA

Figure 7. Two views of the crystal structure for T7 RNA polymerase bound to its promoter (Cheetham et al., 1999), suggesting that duplex DNA cannot continue in a linear trajectory downstream (a) to the right, (b) out of the page) because of steric clash with amino acid residues 595-608 of the polymerase (shown in gold). The template and non-template strands of the DNA are shown in light and dark blue, respectively, the former reaching down towards the active site (red). The recognition loop of the protein is shown in yellow, with the residues contacting the central major groove in green.
bending. They suggest that the energy invested in bending can be used to facilitate base-pair opening, or conversely, disruption of a base-pair can lead to a natural tendency to bend.

Studies of the effects of promoter mutations, Schinkel et al. (1988) showed for the mitochondrial RNA polymerase that the magnitude of enzyme induced promoter-bending correlates with the affinity of the polymerase for the promoter and with the efficiency of transcription. But no direct experimental evidence exists in any system showing that DNA bending, or wrapping of duplex DNA around the polymerase, is required for open complex formation.

Since the DNA helix is disrupted in the melted form of the complex, in the simplest analysis there is no reason for the overall (net) path of the DNA (upstream and downstream) to remain linear. If, however, the DNA starts out linear in the closed complex, then retention of upstream and downstream protein-DNA contacts would be simplest were the net helical trajectory to remain linear. The crystal structure of the polymerase bound to a promoter fragment suggests that the formation of a closed complex cannot occur with completely linear DNA. As illustrated in Figure 7, positioning of fully duplex DNA so as to achieve the strong major groove contacts from positions -10 to -6 (Cheetham et al., 1999; Li et al., 1996) would lead to downstream steric clash with the region of the protein shown in gold. We propose that in order to avoid this clash, the DNA must bend. Indeed, we have recently shown that an energetically unfavorable process is associated with binding of the downstream region of the promoter and that DNA constructs which completely remove this region of the DNA bind to the polymerase more tightly than does full-length DNA, as predicted by this model (Ujvári & Martin, 1997).

These considerations lead directly to a potential mechanistic model for promoter melting. Bending of the DNA near positions -2 to +1, induced by unfavorable steric interactions downstream, would facilitate an overall melting near the start site, as discussed above. Although the sequence TATA adjacent to the start site might facilitate this process (Ujvári & Martin, 1997), it does not seem to be absolutely required for binding and bending. Given the observation that E. coli RNA polymerase also bends its promoter near the start site, it is possible that a similar steric mechanism contributes to the mechanism of melting in this and other more complex RNA polymerases (deHaseth et al., 1998; Perez-Martin et al., 1994).

Materials and Methods

General methods

Oligonucleotides were synthesized by the phosphoramidite method on a Milligen/Biosearch Cyclone Plus DNA synthesizer. Single strands were purified trityl-on using an Amberchrom reverse phase resin as described (Schick & Martin, 1993). Plasmids were propagated in E. coli strains HB101 or XL1-Blue, and were purified on Qiagen columns (Qiagen Inc.)

Restriction enzymes, Klenow fragment of DNA polymerase I, T4 DNA ligase and T4 polynucleotide kinase were obtained primarily from New England Biolabs. Pfu and Taq polymerase were purchased from Stratagene and Gibco-BRL, respectively. T7 RNA polymerase was prepared from E. coli strain BL21 carrying the overproducing plasmid pAR1219 (kindly supplied by F. W. Studier), in which RNA polymerase is expressed under inducible control of the lac UV5 promoter (Davanloo et al., 1984).

PCR samples were separated from the unincorporated nucleotides using a Qiagen PCR purification kit and salts and enzymes were removed from restriction digested samples by the use of a Qiagen nucleotide removal kit.

Following electrophoresis, gels were dried and the radioactivity was quantified using a Molecular Dynamics Storm 840 Phosphorimager.

Plasmid construction

Plasmids pSAU1, pSAU2 and pSAU3 for the circular permutation assay were constructed by inserting oligonucleotides 1, 2 and 3, respectively, shown in Figure 1, between the unique EcoRI and HindIII sites of the permutation vector pSL6 (Gaal et al., 1994). Phasing analysis plasmids pTAU1 to 6, pTAU11 to 16 and pTAU21 to 26 (where the suffix indicates the increasing distance of the promoter from the A-tract), containing the promoter sequence 1, 3 and 4 from Figure 1, respectively, were constructed by cloning the oligonucleotides between the XbaI and SalI sites of plasmids pTK401-26 and pTK401-28 (Kerppola & Curran, 1991). To generate plasmids with different separation between the T7 RNA polymerase promoter and the intrinsic DNA bend, oligonucleotides of different length, containing an additional GACC or GACA/CACAA sequence inserted between the promoter and the intrinsic bend, were used as shown in Figure 4.

Sequences were initially verified by digestion of the plasmids with a restriction enzyme that cuts closely after the promoter sequence, and running a transcription reaction on these fragments with T7 RNA polymerase. Only plasmids carrying the T7 RNA polymerase promoter sequence produced the expected length RNA and were observed on a denaturing acrylamide gel. Mutant promoters were assayed the same way, since they were able to direct transcription at a reduced level. Plasmid were subsequently sequenced within the region targeted for PCR in order to directly verify the correct preparation of the individual constructs.

Preparation of DNA probes for gel shift assay

For the circular permutation assay, radioactively labeled 344 bp DNA fragments were produced by PCR amplification of plasmids pSAU1 through 3 in the presence of [α-32P]dATP using the primers 5′-GGCGTAT-CACGAGGCCCCTTTC-3′ and 5′-ATGGGGTCAGGTGG-GACCAAG-3′. The obtained DNA was digested with different restriction enzymes to generate a set of fragments of equal length (163 bp) containing the promoter sequence at different positions within the restriction fragment (restriction enzymes described by Gaal et al. (1994)).

Intrinsic DNA bend standards containing different numbers of phased A-tracts at the middle or at the end
of the probes were prepared by cutting plasmids pJT170-2 through 5 with NheI or BamH1, respectively (Thompson & Landy, 1988). Molecular mass standards were generated by digestion of pUCM22 (described in Muller et al. (1989)) with the restriction enzyme NheIII. Radioactive labeling of the standards was carried out by filling in the overhanging DNA ends with DNA ligase. 

Probe phasing analyses were prepared by PCR amplification of plasmids pTAU1 to 6, 11 to 16 and 21 to 26 using the primers 5'-TAGGCGTATCAGACGCCCT-3' and 5'-GTAGGCAATTTAGTGTGAT-3'. The resulting DNA fragments were 363 to 373 bp in length and contained the T7 RNA polymerase promoter and the intrinsic bend at the center as shown in Figure 4.

Gel shift assay

Buffered enzyme-DNA complex was prepared by mixing 4 μl DNA (≥5000 cpn) in 10 mM Tris (pH 8.5) buffer with 2 μl of 5× buffer containing 250 mM Tris (pH 8.5), 50 mM MgCl2, 5 mM DTT and 10 μg/ml salmon sperm DNA and with 2 μl of 25% (v/v) glycerol. After incubation on ice, 2 μl of 5× T7 RNA polymerase solution was added in 30 mM Tris (pH 8.5), 100 mM glycine and 0.25% (v/v) Tween-20 buffer. The 10 μl final reaction conditions were: 60 mM Tris (pH 8.5), 20 mM glycine, 10 mM MgCl2, 1 mM DTT, 2 μg/ml salmon sperm DNA and 5% glycerol. Salmon sperm DNA was included in the gel shift buffer to prevent non-specific binding of the polymerase to DNA. Gels were pre-run for three to five hours and the electrophoresis buffer in both reservoirs was replaced with fresh buffer prior to sample loading.

The circular permutation complexes were analyzed by electrophoresis in 8% (w/v) polyacrylamide (acrylamide to bisacrylamide, 60:1, w/w) gels in 4 mM Tris, 160 mM glycine, buffer (pH 7.8) at a field strength of 6 V/cm at 4°C for 15 hours. Phasing analysis probes were run in 6% polyacrylamide gels in 20 mM Tris, 160 mM glycine, buffer (pH 8.5) at a field strength of 12 V/cm at 4°C for five to six hours. Buffers were prepared and the pH was measured on ice. The amount of Tris or the exact pH of the buffer was found not to be important in the gel shift experiments, but pre-running the gel for more than three hours was critical to ensure homogeneous running of all samples in each lane.

Free DNA probes used in Figure 6 were run on a 6% (w/v) acrylamide gel in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA).

Analysis of binding in permutation assays

The mobilities of the free probes and the complexes were measured by the program ImageQuant (Molecular Dynamics) as the distance from the bottom of the loading well to the position of maximal intensity for the corresponding band. In order to calibrate the migration behavior of known bends under our conditions, we compared the mobilities of standard DNAs containing two to five phased A-tracts. A-tracts were assumed to bend DNA toward the minor groove with an estimated 18° per A-tract. The distances the A-tract standards traveled from the top of the gel were fitted to the empirical equation:

\[ \frac{\mu_M}{\mu_E} = \cos \frac{kx}{2} \]  

where \( \mu_M \) and \( \mu_E \) are the mobilities of the fragment with the bend at the middle or at the end, respectively. \( x \) is the net bend angle for the complete A-tract (18° \times \text{number of phased A-tracts}). Kerppola & Curran (1991) introduced the coefficient \( k \) into the original equation of Thompson & Landy (1988) in order to better fit the observed mobilities. The fitted value of \( k \) was 1.0 to 1.2 (depending on gel conditions) was used in equations (3) and (5).

The mobilities of the protein-DNA complexes from the circular permutation assays were normalized to the maximum mobility and fitted to the equation:

\[ \mu_{rel} = A_{CP} \cos \left( \frac{x - \Delta x}{p} \right) + \mu_0 \]  

where \( x \) is the distance in base-pairs of position \( -6 \) from the end of the fragment, \( \Delta x \) is the distance from position \( -6 \) to the center of the bend, \( P \) is the length of the DNA (163 bp), and \( A_{CP} \) is the amplitude. Fitting the permutation data to equation (2) gives the center of the apparent bend at \( x - \Delta x \), and from the permutation amplitude, the estimated angle of the bend (\( 2\alpha_{CP} \)) can be calculated:

\[ \cos \frac{kz_{CP}}{2} = 1 - A_{CP} \]  

Analysis of bending in phasing assays

Mobilities of both protein-bound and free DNAs in the phasing assay were expressed relative to the average mobility and were fitted to equation (4):

\[ \mu_{rel} = \frac{A_{PH}}{2} \cos \left( \frac{S - S_i}{P} \right) + \mu_0 \]  

where \( S \) is the distance of position \( -6 \) from the center of the A-tract, \( S_i \) is the separation that results in maximum mobility, \( P \) is the helical periodicity (set to 10.5), and \( A_{PH} \) is the amplitude. From the fitted phasing amplitude, the apparent angle of the bend can be calculated using equation (5) (Kerppola & Curran, 1991):

\[ \tan \frac{kz_{PH}}{2} = \frac{A_{PH}}{2} / \tan \frac{kz_{C}}{2} \]  

where \( z_{PH} \) is the bending angle to be determined, \( z_{C} \) is the overall bending angle of the three phased A-tracts (54°), and \( k \) is the empirically defined coefficient determined from equation (1).

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