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Bending of synthetic bacteriophage 434 operators by bacteriophage 434 proteins

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ABSTRACT

The extent of DNA bending induced by 434 repressor, its amino terminal DNA binding domain (R1-69), and 434 Cro was studied by gel shift assay. The results show that 434 repressor and R1-69 bend DNA to the same extent. 434 Cro-induced DNA bends are similar to those seen with the 434 repressor proteins. On ~265 base pair fragments, the cyclic AMP receptor protein of *Escherichia coli* (CRP) produces larger mobility shifts than does 434 repressor. This indicates that the 434 proteins bend DNA to a much smaller extent than does CRP. The effects of central operator sequence on intrinsic and 434 protein-induced DNA bending was also examined by gel shift assay. Two 434 operators having different central sequences and affinities for 434 proteins display no static bending. The amount of gel shift induced by 434 repressor on these operators is identical, showing that the 434 repressor bends operators with different central sequences to the same extent. Hence, mutations in the central region of the operator do *not* influence the bent structure of the unbound or bound operator.

INTRODUCTION

In the complexes of the *Escherichia coli* phage 434 operator with phage 434 repressor and Cro, one α-helix of the bound protein dimer lies in each half-site of the operator (1–3). Amino acids side chains protruding from the surface of each helix can contact the outermost five base pairs in one half-site of the 14 base pair operator (2,3). The innermost 2 base pairs in each half-site are not contacted by the proteins (2,3). Nonetheless, both repressor and Cro bind more tightly to operators bearing A·T or T·A base pairs at central operator positions, than to operators bearing G·C or C·G base pairs at these positions (4). The DNA in 434 repressor- and 434 Cro-operator complexes is bent toward the protein and the bases at center of the operator are overtwisted (2,3). These DNA deformations are required for the proteins to make optimal contacts with the base pairs in each operator half-site. We have suggested that the non-contacted base pairs at the center of the operator affect the affinity of the 434 operator for the 434 proteins by influencing either the static structure or the deformability of this region of the operator (4,5). To determine whether or not there is a relationship between DNA bending and the sequence at the center of the operator, DNA bending in the complexes formed between the 434 operator and 434 repressor and Cro is examined.

Gel mobility shift assays can be used to study DNA bending (6). In these assays, the decreased mobility of a DNA fragment containing a putative bending locus near its center, relative to the mobility of the fragment containing this locus near its end, is indicative of DNA bending. Gel mobility shift assays can be used as a quantitative measure of DNA bending (7–9). Using this assay, we have previously detected 434 repressor-induced DNA bending (4). This observation is in agreement with results of x-ray crystallographic investigations (1,2).

The DNA in the crystal structure of a 20 base pair operator fragment in complex with 434 repressor is bent approximately 20–25° (2). Several lines of evidence show that the cyclic AMP receptor protein (CRP) of *Escherichia coli* bends DNA when bound at its binding site (6, 11–13). Results of gel mobility shift assays using >200 base pair fragments, estimate the amount of CRP-induced bending to be at least 90–140° (8). If gel mobility shifts are an accurate reflection of amount of DNA bending, then 434 repressor would be expected to induce a smaller mobility shift than does CRP. Consistent with this prediction, the results in this paper show that 434 repressor does indeed induce smaller mobility shifts than CRP. This affirms the quantitative nature of mobility shift data and places a firm upper limit on the amount of repressor induced DNA bending.

Central base sequence appears to control affinity of 434 operator for 434 proteins by influencing DNA structure (2,4,5). It has been suggested that central base sequence effects on affinity could arise from sequence dependent effects on DNA bending (4,10). If true, central base composition can be imagined to affect the affinity of 434 operator for 434 repressor in any of three ways: 1) by influencing the amount of bending in the free DNA; 2) by limiting the degree that the DNA can be bent in the complex; 3) by altering the ease with which the DNA can be deformed into the properly bent configuration for complex formation. As a first step towards distinguishing between these possibilities, we determined the relative gel mobilities of two 434 operators both alone and in complex with the 434 repressor. Except at the two central base pairs, these operators are identical in sequence. However, their affinity for 434 repressor differs by 50-fold (5). The results of these studies indicate that central base composition affects neither intrinsic bending nor extent of bending in the

protein-DNA complex. Though consistent with the possibility that central base composition affects binding affinity by altering the deformability of the DNA, these results do not establish such a relationship.

METHODS

Binding Sites, Plasmids, DNA Fragments and End-Labelling

DNA manipulations were performed as described (14). The binding site oligonucleotides were synthesized in an Applied Biosystems model 380A DNA synthesizer, gel-purified, annealed and ligated into the unique *Sal* I site in pUC18 (15). The sequence of the CRP binding site used here was A-A-T-T-G-T-G-A-T-C-T-A-G-A-T-C-A-C-A-A-T-T; the sequences of the 434 binding sites used are A-C-A-A-T-A-T-A-T-A-T-T-G-T (reference operator) and A-C-A-A-T-A-G-C-T-A-T-T-G-T (7G operator). For the DNA bending experiments, the approximately 265 base pair circularly permuted fragments containing one synthetic CRP or 434 binding site were constructed in several steps. Each binding site containing pUC18 plasmid derivative was digested with *Bam* HI, followed by repair of the recessed ends with Klenow and deoxynucleotide triphosphates. Following ligation and transformation, the resulting plasmid DNA was digested with *Eco* RI and *Xma* I and the recessed ends repaired to give flush-ended fragments. This linear DNA was ligated, recreating the *Eco* RI site, and transformed. An approximately 270 base pair *Hae* III fragment was gel-isolated from the resulting plasmid and ligated into its parent vector which had been previously cut with *Hind* III, phosphatased and had its recessed ends repaired using Klenow and deoxynucleotide triphosphates. The sequences of the resulting plasmids were confirmed by dideoxy methods. Cleavage of the resultant DNA with *Eco* RI, *Hin* PI, *Ban* I or *Pvu* II results in fragments which have the center of the binding site located approximately 25, 125, 165, and 205 base pairs from one end, respectively (Figure 1). These phosphatased, binding site-containing DNA fragments were gel-isolated and 5'-end labelled using [γ^{32}P]ATP and T_4 polynucleotide kinase. For phase sensitive detection of DNA bending, an isometric set of DNA molecules containing two 434 operators, each having the sequence A-C-A-A-T-A-T-A-T-A-T-T-G-T, were constructed by inserting one operator into each the *Sal* I and *Asp* 718 sites in pUC18. The spacing between the operators was varied over more than one helical turn by digesting and religating the plasmid at restriction sites located between the two operators. The resulting DNA molecules were cleaved at the *Eco* RI and *Hind* III sites and 3'-end-labelled by repairing the recessed ends with Klenow and [α^{32}P]dATP.

Proteins

434 repressor was isolated from the *E. coli* strain X90 bearing a plasmid that causes overproduction of the 434 repressor (16). The 434 Cro and R1-69 proteins were gifts from C.Wolberger and M. Drottar, respectively. CRP was isolated as described by Eileen et al. (17) from a *cya*$^-$ *E. coli* strain bearing the plasmid pHA5 (18).

Non-Denaturing Gel Electrophoresis of Protein-DNA complexes

Binding reactions were performed in 10 mM Tris·HCl, 1 mM $MgCl_2$, 5% (vol/vol) glycerol or sucrose, and either 50 mM (434 proteins) or 200 mM KCl and 200 μM cAMP (CRP). After a 10–20 min incubation at 0°C, the reaction mixtures containing labelled DNA and an appropriate amount of protein were fractionated by electrophoresis at 18 V·cm^{-1} in 8% polyacrylamide gels (29:1 monomer:methylene *bis*acrylamide) containing 90 mM Tris·HCl, 90 mM sodium borate, and 1 mM EDTA at 4°C. The gels were fixed and dried onto Whatmann 3MM paper. An autoradiograph was prepared by exposure of the dried gel to pre-flashed Kodak XAR-5 film with an intensifying screen at −70°C. The relative amount of protein-induced DNA bending is quantified by measuring the mobility the bands corresponding to free and bound DNA using a scanning densitometer. The point of highest intensity was judged to be represent the band center. Pairwise comparisons of extent of protein-induced DNA bending between the various proteins were made by plotting the relative mobility (ratio of bound/unbound mobilities) *versus* the molecular weight for the protein-DNA complexes at each operator position. The slope of the line drawn between the relative mobilities of the two protein-DNA complexes at a single operator position were compared to the slopes of similar plots at another operator position. Significant differences in the slopes of these lines would indicate a difference in amount of DNA bending induced by the two proteins being compared.

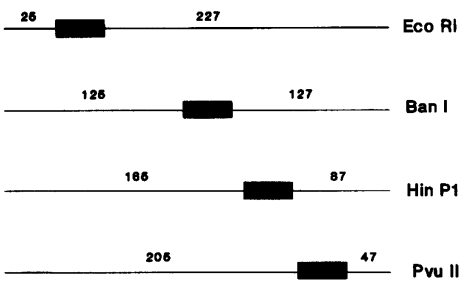

Figure 1. Relative positions of protein binding sites. Plasmids containing circularly permuted fragments bearing synthetic binding sites for the 434 proteins or CRP were constructed as described in Methods. Cleavage of these DNAs with the restriction enzymes shown place the binding site, indicated by filled boxes, at various positions along the DNA fragment. The numbers denote the spacing (in base pairs) separating the edge of the synthetic binding site from the end of the fragment.

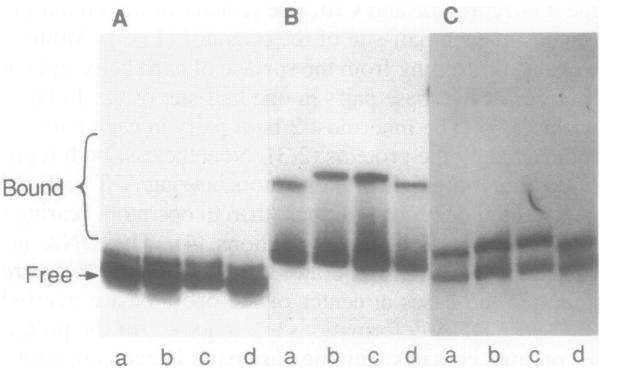

Figure 2. DNA bending by 434 proteins. The amount of mobility shift induced by (A) R1-69, (B) 434 repressor, and (C) 434 Cro on the ~265 base pair circularly permuted operator-containing DNA that has been cleaved with *Eco* RI, lanes a; *Hin* PI, lanes b; *Ban* I, lanes c; or *Pvu* II, lanes d. See also Figure 1. The different panels of the figure depicts one gel, photographed at different exposures.

Phase Sensitive Detection of DNA Bending

Nondenaturing gel electrophoresis of 434 proteins in the presence of the isometric DNA fragments was performed as described above. The amount of protein needed to fill both sites was determined in independent DNAse I footprinting experiments. The complexes which have both operators bound by protein were identified by exposing the DNA-protein complex to DNAse I prior to non-denaturing gel electrophoresis and excising the protein-DNA complexes from the gel. This DNA was purified by electroelution and subsequently electrophoresed on a sequencing gel (data not shown).

RESULTS

A previous investigation of 434 protein-induced DNA bending utilized small (82 base pair) DNA fragments. In that study, the amount of DNA bending was estimated from differences in the relative mobility shifts of just two fragments. Apparently, under certain conditions this protocol limits the degree of observable mobility shift and hence alters the estimated DNA bending (see below). Moreover, the locus of the induced DNA bend could not be determined from such limited data. To further investigate 434 protein-induced DNA bending, new sets of circularly permuted DNAs were constructed, which when cleaved appropriately, would generate approximately 265 base pair fragments bearing operators at four different relative positions (Methods). Figure 2 displays the relative mobility shifts induced by 434 repressor, the amino terminal domain of 434 repressor (R1-69) and 434 Cro on the 265 base pair fragments. An analysis of these mobility shifts (Methods) indicates that the 434 repressor, R1-69 and 434 Cro each bend DNA to the same extent.

An examination of Figure 2 reveals that the 434 protein-induced changes in relative mobility of the fragments bearing the operators at different positions are small. A lingering doubt exists that these mobility shifts reflect the intrinsic effects of varying the position of protein on the fragment and not DNA bending. We tested this by examining the phase sensitivity of the putative DNA bends (19). For this experiment an isometric set of DNA molecules, each bearing two identical operators separated by various spacer lengths, was constructed (Methods). If the protein under examination bends DNA, the orientation of two bends induced by the two bound proteins will vary with the spacing between the two binding sites. As a result, the mobility of the protein-DNA complex should increase from a minimum to a maximum as the protein-induced bends change from being in phase to each other, thereby giving a small end-to-end distance, to being out of phase, giving the largest end-to-end distance. Figure 3 summarizes the results of this experiment for 434 repressor and 434 Cro, analyzed as described by Zinkel and Crothers (19). For both proteins, the mobilities of the protein-DNA complexes are minimal when the two operators are between 22 and 25 base pairs apart and increases to a maximal value when the operators are separated by 17 or 30 base pairs. These data indicate that the 434 repressor and 434 Cro bend DNA. This confirms the conclusions drawn from analysis of gel mobility shift assays (Figure 2).

The periodic oscillation of the mobilities of the protein-DNA complexes can be described by a phase angle of 34°/base pair corresponding to 10.6 base pairs/turn. This periodicity argues that the 434 protein-induced changes in mobility of DNA fragments results from a bend that is directed in space and not from a point of induced bending flexibility. Induced bending flexibility should result in an oscillation of mobility with a period of approximately 5–6 base pairs.

Gel mobility shifts induced by 434 repressor and CRP were compared in effort to estimate the degree of DNA bending induced by 434 repressor. For these experiments two DNA molecules, containing either the 434 operator or a symmetrical consensus CRP binding site were constructed as described in Methods. When cleaved appropriately, these DNAs yield approximately ~265 base pair fragments containing the protein binding site at various positions with respect to the ends (Figure 1). In these DNAs, the binding sites for the two proteins are embedded in identical flanking sequences, hence any differences in mobility shift can therefore only be attributed to the effect of the proteins. Figure 4 shows that the relative mobility shifts induced by CRP on 265 base pair fragments are much larger

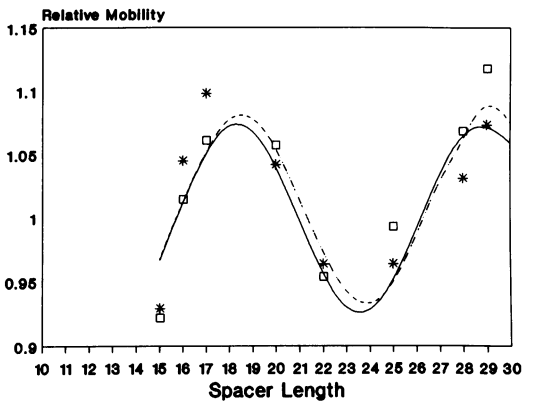

Figure 3. Phase sensitive detection of 434 repressor and 434 Cro DNA bending. The normalized relative mobilities of the protein-DNA complexes containing two bound proteins are plotted as a function of the length of DNA separating the two binding sites. The mobilities of the Cro-DNA and repressor-DNA complexes were first normalized to the molecular weight of the repressor-DNA complex. These mobilities were then normalized to the average mobility of the unbound DNA, as described in reference 19. The asterisks represent the mobilities of the 434 repressor-DNA complexes and the boxes represent the mobilities 434 Cro-DNA complexes. The lines denote best fits to the data assuming 34° and 34.4° for repressor-DNA and Cro-DNA complexes, respectively.

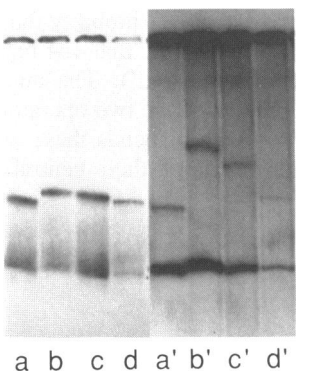

Figure 4. DNA bending by 434 repressor and CRP. Each protein is bound to an ~265 base pair fragment containing a cognate synthetic binding site; lanes a–d, 434 repressor; lanes a'–d', CRP. The fragments were generated by cleaving the circularly permuted binding site-containing DNA with *Eco* RI, lanes a & a'; *Hin* PI, lanes b & b'; *Ban* I, lanes c & c'; or *Pvu* II, lanes d & d'. See also Figure 1.

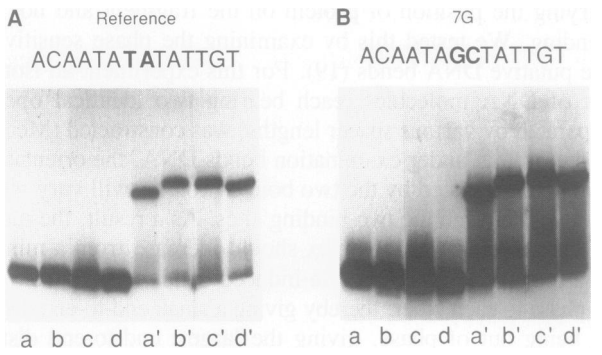

Figure 5. Effect of central operator sequence on DNA bending by 434 Repressor. Circularly permuted DNA containing either the reference operator, (A), or the 7G operator (B) electrophoresed in absence (lanes a−d) or presence (lanes a′−d′) of 434 repressor. The sequence of each operator is given. The central bases which differ between the two operators are highlighted in boldface. The fragments and lane lettering are the same as in Figure 2.

than those induced by 434 repressor. This indicates that CRP bends this DNA to a larger extent than does 434 repressor. Under the conditions of this experiment, no binding to the wild-type *lac* CRP site that is also present on this fragment can be detected (data not shown). Therefore, this larger change in relative mobility shift seen with CRP is not caused by the binding of two CRP molecules to the 265 base pair fragment. Moreover, the locus of bending estimated from the data is located at the synthetic site and not between the two sites. From these data it appears that 434 repressor bends DNA to a significantly lower extent than does CRP.

To investigate whether the central operator sequence affects affinity of 434 operator for 434 repressor by altering the bending of either the unbound or bound operator, the relative mobilities of two different operators were compared in the absence and presence of 434 repressor (Figure 5). The two operators are identical in sequence, except at the centermost two base pairs (Figure 5) and previous work shows that these operators differ in affinity for repressor by 50-fold (5). In the absence of the protein, the relative mobilities of the fragments containing either operator are identical. This indicates that the operators do not contain a static bend (Figure 5 A & B, lanes a−d). When complexed to the 434 repressor, both the reference and 7G-containing operator fragments bearing the operator located near the center of the have a slower mobility than those bearing the operator near its end, indicating that 434 repressor bends DNA (Figure 5 A & B, lanes a′−d′). The amount mobility shift induced by 434 repressor on the two operators is identical. This shows that the 434 repressor bends these two operators to the same extent, independent of their central base sequence or affinity.

DISCUSSION

The relative gel mobility shifts induced by all the 434 proteins, corrected for molecular weight of the complexes are nearly identical. This suggests that these proteins, 434 Cro, 434 repressor, and R1-69, bend DNA to similar extents. In the case of 434 Cro and R1-69, this is consistent with x-ray crystallographic results (2, 20). The locus of bending as determined from the data in Figure 2 is located within or near the binding site on the fragment (data not shown). An analysis of the type of data presented in Figure 3 can place the locus of induced bending more accurately. For both 434 repressor and 434 Cro, the isomer in which the two binding sites are phased to give minimal mobility of protein-DNA complex has the centers of the two dyad symmetric binding sites on the DNA fragment separated by 3.5 helical turns, whereas maximal mobility occurs when the dyad centers are separated by 3 or 4 helical turns. This is an unexpected result. For tandemly repeated binding sites, minimal mobility is expected when an integral number of helical turns separates the binding site centers. This result can be explained in either of two ways. In one case, if the 434 protein-induced DNA bends are not centered at the dyad axis, but instead are (symmetrically) displaced three to four base pairs towards the outer edge of the operator, the two bends in each operator site would be in phase with each other and, moreover, these bends would be separated by integral numbers of helical turns from bends in the second binding site. In an alternative explanation, the minimal mobility observed at half-integral spacing may be a result of a gel electrophoretic chiral selection anomaly of the type recently described by Drak and Crothers (21). While this alternative has merit, the suggestion that the bending loci and operator dyad are not coincident is favored since in addition to accounting for the phase sensitive detection data, this rationale is in excellent agreement with locations of the DNA bending loci seen in the x-ray crystal structures of the 434 repressor and 434 Cro complexes with DNA (2,20). In any case, both the results of the gel mobility shift assays and phase sensitive detection experiments are consistent with the idea that the 434 protein-induced DNA bending originates from protein-DNA contacts localized to a small region of the DNA, within the operator site.

In previous experiments, a mobility shift indicative of Cro-induced bends in DNA was not detected (4). Embedding the 434 operator in longer DNA fragments does allow the ready detection of 434 Cro-induced DNA bending (Figure 2). The assertion that the position dependent effects of Cro binding on mobility of the protein-DNA complex reflects DNA bending is supported by the observed phase sensitivity of this induced mobility shift (Figure 3). A subsequent reexamination of Cro interaction with the smaller DNA fragments also showed Cro-induced DNA bending effects (data not shown). The failure to detect Cro DNA bending in the previous experiments probably results from the low acrylamide concentration in the gels used for those experiments.

Although changing the central base pairs in the 434 operator affects the affinity of operator for 434 proteins, the results biochemical and x-ray crystallographic experiments show that these bases are not in contact with the protein (2,4). These bases are therefore thought to affect operator affinity for repressor by influencing DNA structure and/or flexibility. The bases at the center of the 434 operator are significantly overtwisted and the DNA in the complexes with the 434 proteins is bent. (2,4,20, this paper). Thus, one question at issue is whether the sequence changes at the center of the 434 operator affect affinity for protein by influencing either DNA twist, DNA bend or both. In this paper, the possible relationship between DNA bending and central operator sequence effects on operator strength has been examined.

Gel mobility shifts assays were used to help distinguish between three possible models of how non-contacted bases in the 434 operator could affect affinity by influencing DNA bending. In one model, the central base composition of the different operators would influence affinity for the repressor by altering the degree

of bending of the unbound operators. This postulate predicts that operators with differing affinities would display different degrees of intrinsic bending. To test this idea, the mobilities of two sets of DNA fragments were compared, each set containing an operator bearing a different central sequence. The results show that although these two operators differ in affinity for 434 repressor by 50-fold, neither contains an intrinsic bend.

In an alternative model, variations in base compositions at the operator's center could influence affinity by differentially altering the degree of operator bending in the protein-DNA complex. An effect of this type has been observed with CRP; sequence changes in the 'bending domain' of the CRP binding site affect its affinity by altering the degree of DNA bending. These affinity changes are thought to result from alterations in non-specific DNA contacts (9). Thus, according to this model the amount of DNA bending seen in the complex should depend on the central base composition. The data presented here show that 434 repressor induces the same degree of bend when bound to two operators with different central sequences and affinities.

The above discussions suggest that the central sequences do not affect the degree of operator DNA bending, either alone or in complex with 434 proteins. Thus, *if* the central sequence preferences of the 434 proteins are related solely to DNA bending, as opposed to effects on DNA twisting, then the central bases could be affecting operator bending flexibility. In this case, the affinity differences between operators with dissimilar central sequences would, therefore, result from sequence dependent differences in bending flexibility.

Travers and co-workers have documented the sequence dependence of DNA flexibility (22,23). Based on the data of Travers, Drew et al. have established a correlation between sequence dependent flexure and operator affinity for 434 proteins (24), although their analysis cannot distinguish between torsional and bend effects (25). The data presented here also do not resolve this ambiguity. If central base composition is assumed to affect only DNA bending flexibility, evaluating the size of the repressor-induced DNA bends allows the assignment of an upper limit on the relative differences in bending flexibility of disparate sequences.

The crystal structure of the repressor-operator complex shows that the 20–25° bend in the operator results from the sum of two smaller ~12° bends, one on each side of the operator (2). The loci of these two bends are two- to three-base steps out from the operator's center. CRP bends DNA by at least 90° (8). This large bend angle also appears to result from the sum of smaller bends (9,13). This three- to four-fold difference in bend angle between the two proteins is accurately reflected in the gel electrophoresis assays. Hence, the amount of 434 repressor-induced DNA bending detected by gel electrophoresis and x-ray crystallography are in agreement and indicate that the size of the bend is small. In this light, sequence dependent differences in bending flexibility would have to be very large in order to account for the binding discrimination that arises from differences in base composition in the non-contacted regions of the operator. Specifically, 12° bends occurring at either end of the 7G operator's central sequence, $^{5'}$T-A-G-C-T-A$^{3'}$, must cost ~2 kcal more than identical bends formed at either end of the reference operator's central sequence, $^{5'}$T-A-T-A-T-A$^{3'}$. Unfortunately, the plausibility of these estimated differences DNA bending energies cannot be rigorously evaluated since good measurements of the free energy of sequence dependent of bending flexibility do not exist (26).

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