DNA Bending by the Bulge Defect†

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ABSTRACT: Comparative gel electrophoresis measurements were used to characterize DNA bending in molecules containing an extra adenosine on one strand, the so-called bulge defect. We used oligomers containing A_6 tracts separated from the bulged base by varying numbers of nucleotides to determine the direction and magnitude of the bulge bend. Helix unwinding by the bulge was determined from the electrophoretic anomaly as a function of the size of the repeated monomers. We conclude that the bulge bend is 21° ± 3°, primarily in the direction of tilt away from the bulged base. The total helical advance of the DNA at the bulge site is smaller than would be the case if the complementary T were present, corresponding to an unwinding by 25° ± 6°. These values are in good agreement with the results of NMR and energy minimization studies of the bulged base in double-helical deoxyoligonucleotides [Woodson, S. A., & Crothers, D. M. (1988) *Biochemistry 27*, 3130–3141].

Structures containing bulged nucleotides have been postulated as intermediates in a number of biological processes such as frameshift mutagenesis (Streisinger et al., 1966; Streisinger & Owen, 1985), RNA splicing (Romaniuk et al., 1987), acridine-induced mutations (Ames et al., 1973), and RNA coat protein binding (Drake et al., 1970). Information concerning the shape and physical properties of such putative intermediates can contribute to the development of mechanistic models for the processes concerned.

Several techniques have been used to examine bulged helices. Synthetic models of proposed mutagenic intermediates have been studied by nuclear magnetic resonance spectroscopy (Patel et al., 1982; Morden et al., 1983; Hare et al., 1986; Woodson, 1987; Woodson & Crothers, 1987a,b, 1988), and two double helices containing an extra adenine on one strand have been crystallized (Saper et al., 1986; Miller et al., 1987; Joshua-Tor et al., 1988). We focus here on determining whether the helix axis is bent in a defined manner by the presence of an extra residue and what, if any, change occurs in the helical screw of the molecule. The results agree well with those predicted from NMR-based molecular mechanics calculations for similar sequences (Woodson & Crothers, 1988).

Experimental Design

Small systematic DNA bends add coherently when they are repeated in phase with the double-helix repeat. Our experiments exploit this feature to study an otherwise undetectably small curvature of DNA by nondenaturing gel electrophoresis. Because the observed mobility in the gel depends nonlinearly on DNA curvature (Koo & Crothers, 1988), we obtained an effective amplification of the structural deformation caused by one bending unit. Furthermore, by coupling two different structural motifs to each other, we were able to measure the direction of bending of the DNA helix by one motif relative to the other. The relative angular orientation of the two bends is a function of their linear separation along the helix axis. Minimal gel mobilities are observed when the two bends are in phase, leading to a minimal end-to-end distance (Lerman & Frisch, 1982; Lumpkin & Zimm, 1982; Wu & Crothers, 1984; Slater & Noolandi, 1986). A knowledge of the relative separation along the helix axis which produces maximal curvature allows determination of the relative directions as well as the relative magnitudes of curvature contributed by each motif. Analogous although less systematic experiments of this kind were reported earlier for the complex of *cis*-Pt with DNA (Rice et al., 1988).

The DNA sequences for these experiments (shown in Figure 1) contained a six-base run of dA·dT base pairs, as required for maximum DNA bending (Koo et al., 1986), and an extra adenine at a separate locus on one strand of the DNA duplex. Control sequences included an interrupted A tract which does not induce bending of the helix (Koo et al., 1986). Additional controls included sequences in which the bulged adenine base was placed on the opposite (T-containing) strand, for which we could verify bending in the opposite direction, as expected. All the DNA constructs shared a common sequence at the sites neighboring the bulge, and the overall length was chosen to be an integral multiple of the average helical screw for B-DNA. The sequence was designed to allow only head-to-tail joining of the monomer units, and symmetry was minimized to avoid hairpin formation.

The effect of an extra adenine on the helical screw of the DNA was measured using sequences (Figure 2) in which the relative phase of the bulged A and the A tract was held constant while the overall length of the monomer was varied from 19 to 22 base pairs. In addition, several sequences with a bulged thymidine were used to compare the effect of an extra pyrimidine on one strand with that observed for a bulged purine.

Materials and Methods

All DNA strands were synthesized on an Applied Biosystems 380B automated DNA synthesizer. Syntheses were carried out on a 1-μmol scale using phosphoramidite chemistry with reagents purchased from Applied Biosystems. The crude products were purified either by preparative gel electrophoresis followed by electroelution directly onto DE-52 columns or by reverse-phase HPLC. Gel electrophoresis was done on standard 18 × 40 cm gels in TBE buffer (90 mM Tris–borate/2.5 mM EDTA, pH 8.3) containing 7 M urea. The acrylamide to bis(acrylamide) ratio was 20:1. DNA strands

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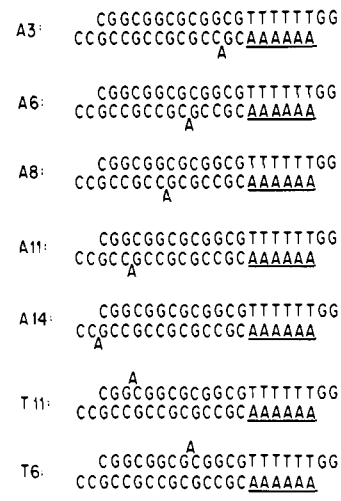

FIGURE 1: Sequences used to measure the direction of bending of a bulged A relative to an A_6 tract. The spacing between the A tract and the bulge is varied over one helical turn. The sequences are named according to the number of base pairs separating the 5′ end of the A tract from the bulge.

FIGURE 2: Sequences used to study the helical periodicity of molecules containing an A tract bend and a bulge defect.

were visualized by UV shadowing and recovered by electroelution.

Preparative HPLC was performed with the trityl protecting group in place on either a Macherey Nagel C_{18} or a Vydac C_4 Protein Pak semi-prep column. Gradients were run in 10 mM triethylammonium acetate, pH 6.8, from 4% acetonitrile to 20–30% acetonitrile, depending on sequence length. The DNA was detritylated by treatment with 80% acetic acid and stored single stranded at −20 °C in unbuffered solutions.

Single-stranded samples were labeled with radioactive phosphorus at the 5′ terminus using T4 polynucleotide kinase (New England Biolabs). Typical concentrations of DNA were (3–7) × 10^{-4} M 5′ ends. Initially, DNA was incubated with 10 μCi of [γ-^{32}P]ATP (Amersham), and the concentration of ATP was then raised to 5 mM to ensure that all 5′ ends were phosphorylated. Double-stranded, blunt-ended samples of *Hae*III-cut ΦX174 were radioactively labeled according to standard protocols and used as size standards.

Equal volumes of complementary strands were combined to give a total reaction volume of 10 μL. The DNA was heated to 80–90 °C and allowed to cool slowly in a water bath to 5–10 °C. The solution was brought to a final volume of 20 μL, and 800 units of T4 DNA ligase (New England Biolabs) were added. Reactions were quenched by addition of EDTA to a final concentration of 15–20 mM after 16–24 h. Samples were analyzed on 8% polyacrylamide gels [29:1 acrylamide:bis-(acrylamide) ratio; 18 cm × 40 cm × 0.3 mm]. The casting and running buffers were 90 mM TBE. Gels were run at room temperature at 2.5–3.0 W until the bromphenol blue marker reached 23 cm from the base of the wells. Gels were fixed in 10% methanol/10% acetic acid for 15 min, transferred onto Whatman filter paper, and dried at 80 °C for 1.5 h. Exposures were overnight on a Dupont Lightening Plus screen using Dupont Cronex X-ray film. Film was developed with a Kodak GBX developer and approximately 90-s developing time.

The distance migrated by standard restriction fragments of known length was measured and fit to an exponential function using least-squares analysis. The apparent molecular weight of sample bands was then calculated according to the function derived and the distance migrated.

Results and Discussion

To determine the direction of the bend at the bulge base, we needed to know the exact phasing between the reference bend of the A tract and the unknown bend to be measured. This requires knowledge of the helical repeat of the DNA. Kabsch et al. (1982) have calculated twist angles for each dinucleotide step in naturally occurring DNA (1982). Applying these values to our sequences lacking the bulge, we obtain a value of 10.4 base pairs per turn for all of them. Various experimental techniques have been used to measure the helical screw of DNA molecules (Wang, 1979; Rhodes & Klug, 1980; Peck & Wang, 1981; Tullius & Dombrowski, 1985). We chose to study the variation of bending anomaly with sequence repeat.

Because oligomers containing bulges close to the ends of the fragment do not ligate well, our bulge-containing molecule is limited to one bending unit every two turns of the helix. This results in a fairly low curvature of the DNA. The relative mobility of bent DNAs varies with the square of the DNA curvature (Koo & Crothers, 1988), and thus, although the sequence with one bulged A per two helical turns does migrate more slowly in the gel (Rice, 1987), the effect is small and precludes an accurate determination of the helical screw through variation of the monomer lengths.

In order to enhance the sensitivity of our experiment, we increased the curvature and hence the gel anomaly effect by constructing a sequence containing the bending motif of interest, a bulged adenine, and a constant bending motif held at near perfect phase to the bulge. The A tract is present in this experiment for the sole purpose of increasing the overall curvature of the DNA. Its position relative to the bulge is irrelevant as long as the spacing is held constant between the different samples. A small error is introduced by this method because the A tract does not remain at constant phase relative to the bulge as the repeat distance (monomer length) is varied. However, the main effect of altered monomer length is to dephase the overall bends, to a degree dependent primarily on the overall helical repeat.

The relative phase of the known (A tract) and unknown (bulge) bends for this experiment was set constant at the position which provides for the greatest anomaly in electrophoretic mobility, determined by experiments described below. The monomer length was then systematically varied from 19 to 22 base pairs both for the parent molecule with only the A tract induced bend (Figure 3) and for the molecule containing both an A tract and a bulge phased in such a way that the bends induced by each were additive (Figure 4). The helical screw was determined by finding the monomer length which corresponded to the greatest anomaly, in which the bends are phased most exactly with each other. We found that the helical screw was close to 10.5 for the A tract alone, although clearly slightly less than 10.5 since the 20 bp repeat was more anomalous in mobility than the 22 bp repeat (Figure 3). In addition, the helical screw must be closer to 10.5 than to 10, since molecules with a 10.5 bp sequence repeat cyclize much more rapidly than do those with a 10.0 bp repeat (Koo

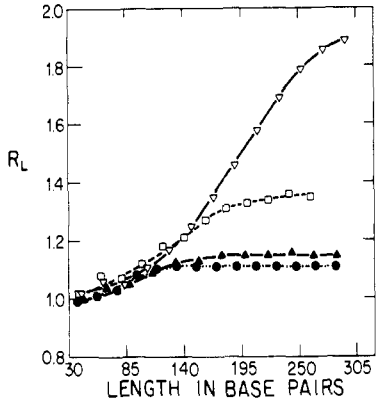

FIGURE 3: Relative mobilities of sequences of different monomer length containing one A_6 tract. The repeat distance was varied from 19 to 22 bp. (●) Multimers of a 19 base pair fragment; (□) multimers of a 20 base pair fragment; (▽) multimers of a 21 base pair fragment; (▲) multimers of a 22 base pair fragment. The anomaly is maximized for the $(21)_n$ fragments, but $(20)_n$ is more anomalous than $(22)_n$.

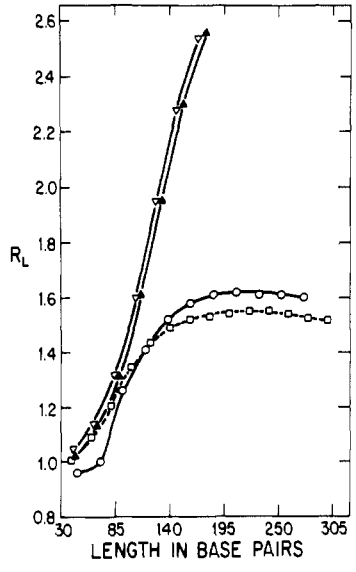

FIGURE 4: Mobilities of 19–22 bp sequences containing a bulged adenine and an A_6 tract (see Figure 2) measured relative to *Bam*HI linkers. (○) 19 base pair monomer fragment; (□) 20 base pair monomer fragment; (▽) 21 base pair monomer fragment; (▲) 22 base pair monomer fragment. The mobilities of the $(21)_n$ and $(22)_n$ fragments are almost identical.

et al., 1986, 1989). We thus set the helical screw equal to 10.4 ± 0.1 bp per turn, for the sequence carrying only an A tract.

The data for molecules which include the bulge show that a 21 base pair repeat and a 22 base pair repeat (counting the bulge) are nearly identical (see Figure 4). We thus set the helical repeat for these molecules equal to 10.75 ± 0.05 base pairs per turn. Since there is only one bulge in two turns of the helix, the entire shift in helical screw of 0.7 bp (21.5–20.8 is equal to 0.7 ± 0.15 base pairs) is attributed to unwinding by the extra adenine. In summary, deletion of the T nucleotide complementary to the bulged A results in helical unwinding by 0.7 bp or 25° ± 6°.

Figure 5 shows the mobility data for the set of molecules in which the phasing was varied between the bulge and the A tract. Clearly, there is a large difference in mobility between isomers in which the bulge and A tract are in phase (A6 and T11; see Figure 1) and out of phase (A11, T6). Since the A tract bend direction can be approximated by tilt toward the A-containing strand at the 3′ junction of the A tract (Koo et al., 1986; Zinkel & Crothers, 1987; Koo & Crothers, 1988), the in-phase relationship when the bulge is approximately

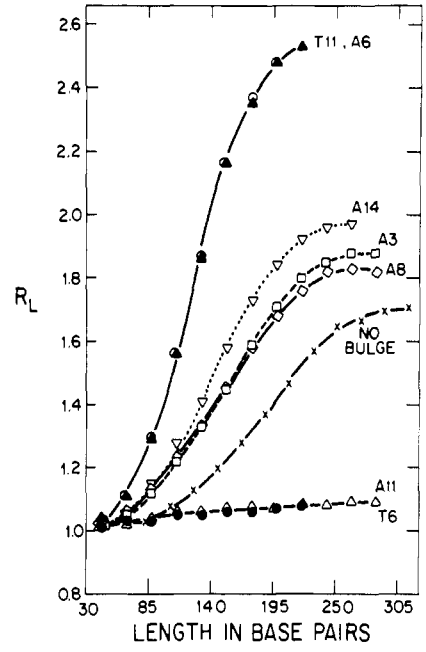

FIGURE 5: Relative mobilities of sequences with different relative spacings between an A_6 tract and an A bulge. The open symbols correspond to the bulge on the A_n strand, and the closed symbols correspond to the bulge on the T_n strand. In all cases, the sequences are named for the number of base pairs separating the bulge from the 5′ junction of the A tract on the A_n strand and correspond to the sequences shown in Figure 1. The control fragments, T6 and T11, behave as predicted: their mobilities match the mobility of sequences where the bulge is on the opposite strand but displaced by a half helical turn.

one-half helical turn from the 3′ junction (A6) indicates that the direction of the bulge bend can be approximated by tilt away from the bulged base, which is on the A-containing strand. Since the out-of-phase A11 isomer is nearly normal in mobility, we can conclude that the bulge and A tract bends are approximately equal in magnitude.

Bending predominantly in the direction of tilt is also required by the observation that T11 and A6 have equivalent bends (Figure 5): moving the bulge to the opposite strand requires that it be displaced by half a helical turn (5 bp) in order to yield in T11 the same bend as in A6. If the bend were in the direction of roll, bending equal to that in A6 should have resulted for the isomer (T6) in which the bulge is transferred to the other strand without helical displacement. Hence, these experiments lead, independently of the A tract bending model, to the conclusion that bending at the bulge defect is in the direction of tilt in a coordinate frame centered at the bulged base. The self-consistency of the set of results further supports the plane (orientation) of bending of the A tract deduced from earlier experiments (Koo et al., 1986).

Adopting the convention that the bend caused by the bulge is viewed in the coordinate frame at the site of the extra base, we are now in a position to calculate more precisely the direction of the bulge bend relative to the standard bend caused by the A tract. This was done by calculating the path of the DNA molecule through space for every possible relative phase of the two bends (over one full turn of the double helix). The calculations used an algorithm previously described by Levene and Crothers (1983). In order to eliminate the effects of overall dephasing, it was necessary to adjust the theoretical helix screw to be such that the 22-base monomer units were a perfect integral multiple of the helical repeat. The parameters used were found by increasing the helical screw (by 4%) for each segment of the DNA by the same proportion until

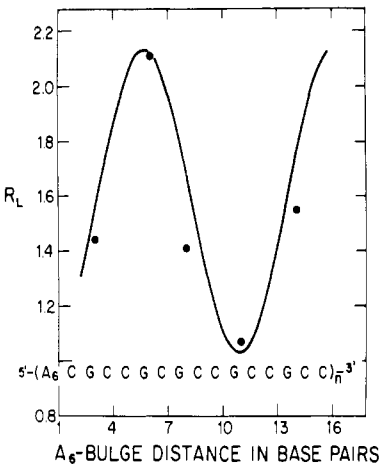

FIGURE 6: Relative mobilities of 150 base pair hypothetical DNA fragments corresponding to the multimers of the sequences shown in Figure 3. The mobilities were obtained by interpolation from the data in Figure 5. The points represent sequences with a bulged A at varying positions relative to the A tract. The curve represents the relative mobilities predicted by the junction bending model where the bulged A is modeled to bend the helix axis by 20° of tilt toward the unbulged strand and by 8° of roll toward the minor groove.

the trajectory lay in a plane for the parent molecule containing only an A tract. After the calculation was completed, the helical screw was readjusted to properly reflect the true repeat of the DNA.

The A tract was treated as a poly(dA)·poly(dT) segment with a helical screw of 10.1 base pairs per turn [see Saenger (1984) and references cited therein]. The helical repeat for B-DNA was taken as 10.5 bp/turn, and the extra base was considered to underwind the DNA by 0.7 bp, relative to the structure in which the extra base has a complement on the other strand. Unwinding was restricted to the two helical steps on either side of the bulge (the total helical advance across the bulge is equivalent to 1.3 bp). The calculated end-to-end distance for the optimal phasing was minimized by adjusting the bend angles for tilt and roll at the extra base. The first minimum in the end-to-end distance was chosen as the optimum circle size and compared to that calculated for $(N_2A_6N_5A_6N_2)_n$. The ratio of the circle sizes was set equal to the relative curvature of the two sequences. Assigning a curvature of 0.7 to the DNA sequence containing one A_6 unit per turn, the relative curvatures are entered into the empirical relationship of Koo and Crothers (1988), $R_L - 1 = (9.6 \times 10^{-5})(L^2 - 0.47)C^2$, to yield relative mobilities, where R_L is the ratio of apparent DNA size on the gel to the real size. L is the actual DNA length in base pairs, and C is the curvature of the DNA. The A tract relative curvature of 0.7 was derived experimentally from the properties of control sequences containing one A_6 tract and no bulge in 21 bp by comparing the gel mobility to that for the sequences for which the equation was developed (Koo & Crothers, 1988). [The flanking sequence for the A_6 unit studied here is different from that of Koo et al. (1986) and is probably responsible for the difference in curvature.] Relative values were assigned by using the junction model to fit gel mobility data for A tracts, and from the modeling of cyclization kinetics (Koo & Crothers, 1988; Koo et al., 1989).

The best values for the angular parameters at the bulge site were found to be 8° of roll toward the major groove and 20° of tilt toward the opposite strand from that carrying the extra base. (This result uses 22° as the overall bend of an A_6 tract.) Figure 6 shows the calculated mobilities for 21 base pair sequences containing both an A_6 bending unit and an extra adenine on one strand of the double helix. The ~10 bp periodicity observed eliminates models in which the bulge forms a hinge instead of a static bend (Zinkel & Crothers, 1987).

In summary, we find that a bulged adenine bends the helix axis principally by tilt toward the opposite strand. For our sequence, we measure 20° of tilt and 8° of roll for a total bend angle of 21° ± 3°; the main source of uncertainty is the possible error in the A tract bend angle (Koo et al., 1989). The missing complementary base at the bulge site unwinds the helix such that, if treated locally, the total helical advance across the bulge is 45° ± 6°, rather than the ~70° expected for two base pairs and a helical screw of 10.5 bp per turn. According to this result, the bulged site corresponds to two steps in a helix of 16 base pairs per turn. Molecular mechanics calculations, based on NMR studies of a short oligomer containing an extra adenine on one strand flanked by the same two base pairs as the sequence studied here, are consistent with a structure in which the helix bends at the bulge site by 18–23° (primarily by tilt away from the extra A) and has a total helical advance of 54° (Woodson & Crothers, 1988). These values are in good agreement with those we have measured by the gel method.

Changing the ratio of tilt to roll is equivalent to moving the site of the bulge-induced bend. A defined ratio of tilt to roll simply defines a single axis in the plane of the base pair about which the rotation occurs. Changing the direction of this axis by changing the ratio of tilt to roll is geometrically equivalent to rotating the base pair about the helix axis. Such rotations are the natural consequence of translation along a helix axis. As we cannot determine both the position and the direction of the bend in this experiment, we report the direction of the bend in a coordinate frame centered on the bulge position. If the actual bend is displaced along the helix axis, the roll and tilt components in the *local* frame are changed accordingly.

NMR and other data suggest that although an extra purine on one strand of a double helix stacks into the helix, a pyrimidine in the same environment spends substantial time outside the helix (Morden, 1983; Patel et al., 1987; Lomant & Fresco, 1973). We therefore examined three sequences containing an extra thymine. (Since one of the bases neighboring the bulge is a cytidine, the only possible choice was a thymine.) We found that the bulged thymine bends the DNA. Although we did not explore a full turn of the helix, we located the maximum at roughly the same position as previously found for an A bulge and showed that the relative mobilities of oligomers with the bulge at positions 5 bp apart are not the same; hence, once again, the additional base bends the DNA in a defined direction. The smaller magnitude of the anomaly represents a smaller average induced curvature of the DNA which may be interpreted as the base spending less time stacked in the helix as is indicated by the NMR data. This interpretation is dependent on the assumption that a nucleotide which is looped out does not kink the helix axis. The exact angle of the bend could not be calculated because we did not measure the helical screw for the T bulge sequences.

Recent crystallographic evidence (Joshua-Tor et al., 1988; Miller et al., 1988) indicates that a bulged purine is extrahelical in the crystalline state, yielding molecules which are nearly straight. Miller et al. (1988) propose that the difference between solution and crystal resides in the better solid-state packing of liner arrays of oligonucleotides when kinks are avoided. Our results, which substantiate a helix bend in solution, support this general interpretation.

Nondenaturing gel electrophoresis is a simple, rapid, and sensitive assay for DNA curvature. In conjunction with ap-

propriate mathematical models, it can be used to provide detailed information about the type, magnitude, and direction of structural distortions in a DNA helix which result in changes in the average end-to-end distance of the molecule. Relatively small amounts of material are required for such experiments, and as the library of synthetic DNA sequences grows, we can expect to increase our understanding of the sequence dependence of DNA structure and our knowledge of local variation from the average B-DNA double helix. The role of local structural variation is unknown but may be potentially significant in protein DNA recognition and binding affinity.

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