Comparison of DNA Bending by Fos−Jun and Phased A Tracts by Multifactorial Phasing Analysis

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ABSTRACT: Studies of DNA bending by Fos and Jun using different methods have yielded contradictory results. Whereas gel electrophoretic phasing analysis indicates that Fos and Jun bend DNA, results obtained through X-ray crystallography and ligase-catalyzed cyclization suggest that they do not. To test the assumptions underlying phasing analysis and to examine DNA bending by Fos and Jun, a multifactorial phasing analysis approach based on the distinct electrophoretic mobilities of DNA fragments of diverse shapes was developed. In this approach, the spacing between the bends, the length of sequences flanking the bends, and the acrylamide concentration in the gel are varied. Two closely spaced intrinsic bends with long flanking sequences had the same effect on electrophoretic mobility as a single bend corresponding to the sum of the bends when they were arranged in phase, and the difference between the bends when they were arranged out of phase. Based on the phase-dependent electrophoretic mobility variation of fragments containing intrinsic DNA bends of different magnitudes, three criteria for determination whether the phase-dependent mobility variation of protein−DNA complexes is caused by DNA bending were adopted. Complexes formed by the bZIP domains of Fos and Jun fulfilled each of these criteria. First, the electrophoretic mobility variation induced by Fos and Jun was proportional to that caused by an intrinsic bend over a broad range of acrylamide concentrations. Second, the mobility difference between fragments containing in phase and out of phase bends was reduced by an increase in the separation between the bends. The separation between the bends had the same effect on the electrophoretic mobility variation caused by Fos and Jun as well as intrinsic bends on long DNA fragments at low acrylamide concentrations. Third, on short DNA fragments analyzed at high acrylamide concentrations, two intrinsic bends separated by long spacers caused a larger decrease in electrophoretic mobility when they were out of phase than when they were in phase. This reversal of the phase dependence of the electrophoretic mobility variation was also observed for complexes formed by truncated Fos and Jun. Thus, the phase-dependent mobility variation of Fos and Jun complexes is due to DNA bending.

The quantitative analysis of protein induced DNA bending is an important tool for determination of the path of the DNA helix in multiprotein complexes involved in the regulation of transcription, replication, recombination, and DNA repair. Many approaches have been used to investigate DNA bending by sequence-specific DNA binding proteins. These include high-resolution structural studies employing X-ray crystallography and nuclear magnetic resonance (Schultz et al., 1991; Love et al., 1995), direct visualization by electron spectroscopic imaging and electron and atomic force microscopy (Bazett-Jones et al., 1994; Erie et al., 1994; Becker et al., 1995; Griffith et al., 1995), topological methods based on the rate of ligase-catalyzed cyclization or the change in linking number (Kahn & Crothers, 1992; Lutter et al., 1996), hydrodynamic measurement of rotational diffusion by transient electric birefringence or electric dichroism (Hagerman, 1984; Levene et al., 1986), spectroscopic techniques utilizing resonance energy transfer (Heyduk & Lee, 1992) and gel electrophoretic methods employing either circular permutation (Wu & Crothers, 1984) or phasing analysis (Zinkel & Crothers, 1987; Kerppola & Curran, 1991a,b). Among these approaches, the gel electrophoretic methods have been most widely employed, in part due to their ease of use and the lack of a requirement for specialized equipment.

The mobility of a DNA fragment during polyacrylamide gel electrophoresis is determined by the shape and size of the fragment in conjunction with gel composition and electrophoresis conditions. The effect of DNA shape on electrophoretic mobility has been used to develop several assays for DNA bending (Marini et al., 1982; Wu & Crothers, 1984; Hagerman, 1985; Zinkel & Crothers, 1987; Kerppola & Curran, 1991a,b). These assays have been applied to the study of both intrinsic as well as protein-induced DNA bends. The first such assay to be developed, known as circular permutation analysis, is based on the lower electrophoretic mobility of DNA fragments containing a bend at the center compared with DNA fragments containing the same bend at either end (Wu & Crothers, 1984). This method has allowed identification of sequences that cause intrinsic DNA bending and localization of the center of such bends. However, circular permutation analysis of protein−DNA complexes can produce erroneous results (Gartenberg et al., 1990; Kerppola & Curran, 1991b, 1993; Kuprash et al., 1995). It is likely that in these situations, the structure of the protein itself has different effects on the electrophoretic

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mobilities of the protein−DNA complexes when bound to the center as opposed to the end of a DNA fragment.

To circumvent the limitations of circular permutation analysis and to obtain additional information about the structure of DNA bends, alternative gel electrophoresis strategies that are based on the phase-dependent interaction between two bends on the same DNA fragment have been devised (Zinkel & Crothers, 1987; Kerppola & Curran, 1991a,b). In phase-sensitive detection (Zinkel & Crothers, 1987), the bend to be examined is placed at different distances from a reference bend of known magnitude and direction. When the two bends are in phase, the fragment is predicted to assume the shape of the letter C, which is thought to result in slower migration than when the bends are out of phase, and the fragment is predicted to assume the shape of the letter S. Although the effect of a single contiguous bend on the mobility of a DNA fragment has been extensively investigated (Wu & Crothers, 1984; Thompson & Landy, 1988; Zinkel & Crothers, 1990), the effect of two separate DNA bends on fragment mobility is not well understood.

To reduce potential complications arising from the presence of multiple bends on the DNA fragment, the reference and test bends were placed immediately adjacent to each other in phasing analysis (Kerppola & Curran, 1991a,b). Because of the close proximity of the two bends, it was reasoned that their effects on electrophoretic mobility would be comparable to the effects of single DNA bends, corresponding to the sum of the two bends when they were in phase, and the difference between the bends when they were out of phase. However, this fundamental assumption of phasing analysis has not been directly tested. Furthermore, several proteins that cause a phase-dependent mobility variation (Kerppola & Curran, 1991a,b; Wechsler & Dang, 1992; Fisher et al., 1992; Natesan & Gilman, 1993) do not bend DNA in crystals (Ferre-D'Amare et al., 1993, 1994; Glover & Harrison, 1995; Houbaviy et al., 1996) and do not increase the rate of ligase-catalyzed cyclization (Sitlani & Crothers, 1996; McCormick et al., 1996). It has therefore been suggested that protein structures unrelated to DNA bending can cause a variation in complex mobilities in phasing analysis.

The phase-dependent mobility variation has been examined in greatest detail for complexes formed by Fos and Jun family proteins (Kerppola & Curran, 1991a,b, 1993, 1997; Paolella et al., 1994; Leonard et al., 1997; Rajaram & Kerppola, 1997). Fos−Jun heterodimers and Jun homodimers cause electrophoretic mobility variations of opposite phase, consistent with opposite directions of DNA bending (Kerppola & Curran, 1991a,b). Heterodimers between proteins that induce equal bends of opposite directions cause no electrophoretic mobility variation (Kerppola & Curran, 1991b, 1997). Single amino acid substitutions alter the magnitude of the DNA bend angle in direct proportion to the change in net charge (Leonard et al., 1997). Reversal of the orientation of heterodimer binding to the AP-1 site causes a shift in the phase of the mobility variation, indicating a change in DNA bend direction (Leonard et al., 1997; Rajaram & Kerppola, 1997). Fos and Jun induce distinct DNA bends when they bind to different AP-1 sites, and substitutions in flanking sequences have converse effects on bending in opposite directions (Rajaram & Kerppola, 1997). Substitution of a single base pair that is not contacted in the X-ray crystal structure alters DNA bending by Fos and Jun (Leonard et al., 1997). These results, in aggregate, strongly support the conclusion that Fos and Jun induce DNA bending at the AP-1 site.

To test the validity of the assumptions underlying phasing analysis, the electrophoretic mobilities of DNA fragments containing two intrinsic DNA bends in different spatial arrangements were determined under various electrophoresis conditions. Based on the mobility variation of these fragments, three criteria were adopted for determination of whether the phase-dependent mobility variation of protein−DNA complexes is caused by DNA bending. Complexes formed by truncated Fos and Jun had the same effect on the electrophoretic mobility variation as intrinsic DNA bends under a variety of conditions, fulfilling each of the criteria. The results demonstrate that Fos and Jun bend DNA and show that multifactorial phasing analysis can be used to determine whether phase-dependent variation in the electrophoretic mobilities of protein−DNA complexes is caused by DNA bending.

EXPERIMENTAL PROCEDURES

Preparation of DNA Fragments and Protein Purification. DNA fragments containing two intrinsic bends, each consisting of three phased A tracts, separated by spacers of different lengths were prepared by polymerase chain reaction (PCR)[1] amplification using plasmids pTK430-14 through pTK430-61 as templates (Kerppola, 1996). To substitute one of the bends on these fragments with a single A tract or an AP-1 site, a two-stage PCR strategy was used. In the first stage, the inside primers CTAGATGGCAAAAACGGGTCGCCGGCGCCCCGGCTC and GGCGACCCGTTTTTGCCATCTAGAGGATCCCTCGAT or CTAGATGCTGACTCATTGTCGCCGGCGCCCCGGCTC and GGCGACAATGAGTCAGCATCTAG were used in separate reactions with the outside primers described previously (Kerppola & Curran, 1991a) to amplify fragments from these templates. In the second stage, the purified products from the first stage were mixed and amplified using the outside primers. Thus, these DNA fragments differ only at the underlined base pairs, at which either an AP-1 site or an A tract centered at the same position was inserted. To prepare fragments with shorter flanking sequences, the PCR products were digested with *Pvu*II or *Nco*I. Fragments containing single DNA bends of different magnitudes were prepared by PCR amplification using plasmids pJT170-2, -3, -5, -6, -7, -8, and -9 as templates (Thompson & Landy, 1988). To prepare fragments with the bend located at the center or toward one end, the PCR products were digested with *Nhe*I or *Bam*HI, respectively. To prepare fragments with shorter flanking sequences, the PCR products were digested with *Taq*I and *Ban*I.

Truncated and full-length Fos and Jun proteins were expressed as hexahistidine fusions in *Escherichia coli* and purified by nickel chelate affinity chromatography as described (Kerppola & Curran, 1991a).

Determination of Electrophoretic Mobilities. Heterodimers between Fos and Jun proteins were prepared by incubation

[1] Abbreviations: bp, base pair(s); bZIP, basic and zipper regions; PCR, polymerase chain reaction; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; DTT, dithiothreitol; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; RT, room temperature.

of a 2-fold molar excess of Fos with Jun in 20 mM HEPES (pH 7.6), 100 mM KCl, 1 mM EDTA, 5 mM DTT, 5% glycerol, 0.1% NP-40, and 0.5 mg/ml BSA to ensure quantitative heterodimerization. Protein−DNA complexes were formed by incubating the proteins with the DNA fragments in the same buffer in the presence of 0.02 mg/mL poly(dIdC) for 5 min at RT. The protein concentrations were adjusted such that approximately 50% of the DNA probe was bound. The mobilities of the DNA fragments and the protein−DNA complexes were analyzed in parallel by electrophoresis in 20 cm × 30 cm × 1.5 mm gels containing the polyacrylamide [29:1 acrylamide:bis(acrylamide) ratio] concentrations indicated in 25 mM Tris, 195 mM glycine buffer at a field strength of 10 V/cm. The gels were run for 1 h prior to loading, and the time of electrophoresis was adjusted such that the DNA fragments and protein−DNA complexes migrated a distance that allowed accurate measurement of differences in their electrophoretic mobilities.

Quantitative Analysis of Electrophoretic Mobilities. The mobilities of fragments containing two intrinsic bends with three A tracts each as well as fragments containing a single bend were compared with the mobilities of 123 bp ladder standards (Gibco-BRL) and with *Hae*III-digested pBR322, and the mobility anomaly was calculated from the ratio between the actual length of a fragment and its apparent length based on its electrophoretic mobility. The mobilities of the protein−DNA complexes as well as the DNA fragments containing a single A tract bend were corrected for differences in the mobilities of DNA fragments containing an AP-1 site and normalized to an average mobility of 1. The mobility anomalies and the relative complex mobilities were plotted as a function of the separation between the centers of the intrinsic bends and the AP-1 site, respectively. These mobilities were fitted to a harmonic oscillation function with exponential decay, designated the damped phasing function:

$$\mu = \mu_0 + kS + A_{0_S} 2^{-(S/S_{1/2_S})} \cos\left(2\pi \frac{S - S_{T_S}}{P}\right) + A_{0_L} 2^{-(S/S_{1/2_L})} \cos\left(2\pi \frac{S - S_{T_L}}{P}\right) \quad (1)$$

in which μ is the observed relative mobility, μ_0 is the relative mobility at conceptual zero separation (in reality, the bends cannot overlap, and therefore μ is never equal to μ_0; this definition of μ_0 is chosen to make the equation general for DNA bends of different lengths), k is the average mobility change per base pair (used to adjust for the larger effect of the length of the DNA fragment on the absolute mobilities of the DNA fragments alone than on the protein−DNA complexes), A_0 is the amplitude at conceptual zero separation, S is the separation for DNA fragment analyzed, $S_{1/2}$ is the separation that reduces the amplitude by half (damping coefficient), S_T is the separation that results in maximum mobility (at this separation, the bends are perfectly out of phase), and P is the helical periodicity of DNA separating the bends. The first and second terms were adjusted to account for the phase-independent change in average mobility with increasing separation. These phase-independent terms account for changes in mobility that are caused by the differences in size of the complexes resulting from the change in the length of the spacer between the bends. The variables in the third term (sub-subscript S) were adjusted to fit the mobilities of fragments with a short separation between the bends. The fourth term was used only for complexes with a change in the phase of the mobility variation of fragments with a long separation between the bends, and the variables (sub-subscript L) were adjusted to account for this change. Although this function contains nine independent variables, they are all well-defined because of the large number of data points, and since only a subset of the variables were adjusted to fit different portions of the data.

The DNA bend angle induced by each complex was calculated based on the ratio between the amplitudes of the mobility variation induced by the complex and that caused by the intrinsic A tract bend at a separation of 21 bp. When the centers of the bends are separated by 21 bp, designated the merge separation, the single A tract test bend and the reference bend consisting of three A tracts merge to form one contiguous bend. Thus, the amplitude of the mobility variation of fragments containing two bends at merge separation is directly comparable to the mobilities of fragments containing single bends of different magnitudes. The merge separation depends on the magnitudes of the two bends. For the fragments containing two intrinsic bends, each consisting of three A tracts, the merge separation is 31 bp. Since the magnitudes of the bends induced by the Fos and Jun proteins examined here are of the same order as that induced by a single A tract, a merge separation of 21 bp was used for these complexes. The amplitude at merge separation (A_{21_S} or A_{31_S}) was calculated based on eq 1.

The mobility anomalies of DNA fragments containing a single bend were plotted as a function of the number of A tracts. These mobility anomalies were fitted to the empirically derived function:

$$\frac{l_{seq}}{l_{gel}} = a\left(1 - \frac{n^b}{n^b + c^b}\right) \quad (2)$$

in which l_{seq} is the length of the fragments based on its sequence, l_{gel} is the apparent length based on electrophoretic mobility, n is the number of A tracts, and a, b, and c are scalars. Since the relationship between DNA bend angle and mobility anomaly for bends consisting of two to four A tracts was nearly linear, the DNA bend angle was assumed to be directly proportional to the amplitude of the mobility variation at merge separation. The maximum error introduced by this approximation within this range is a 2% deviation in the DNA bend angle. The absolute DNA bend angle was therefore calculated from the ratio between the amplitude of the mobility variation induced by protein binding and that caused by an intrinsic A tract bend at merge separation. The DNA bend angle induced by an A tract has been estimated to be 18° based on several independent methods [as referenced in Crothers and Drak (1992)]. The absolute DNA bend angles calculated based on this approach depend on the accuracy of the estimated magnitude of the A tract bend, and are subject to revision of this value. However, the relative DNA bend angles are virtually unaffected by moderate changes in this estimate. Quantitation of the magnitude of DNA bending by phasing analysis also depends on the assumption that intrinsic and protein-induced DNA bends of the same magnitude have identical effects on electrophoretic mobility. Although this

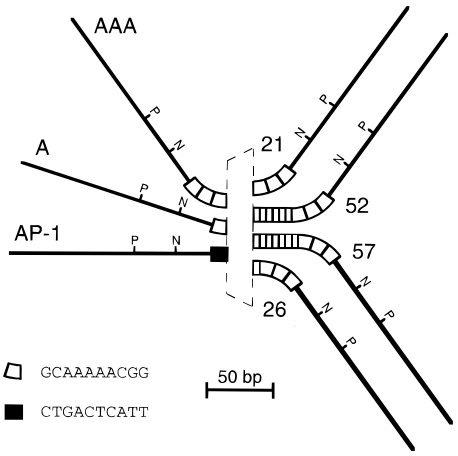


FIGURE 1: Schematic diagram of representative DNA fragments used in multifactorial phasing analysis. The DNA fragments consisted of multiple combinations of the test bends shown to the left of the dashed lines, and the spacers and reference bends shown on the right. The AP-1 site is shown as a solid rectangle, intrinsic A tract bends are shown as open wedges, and spacers corresponding to half a helical turn are shown as open rectangles. The sequences of the AP-1 site and the A tract shown at the bottom represent the only difference between DNA fragments containing the same spacer. The separations between the centers of the AP-1 site and the reference bends are shown next to each reference bend. These separations are identical when the single A tract is fused to each of the reference bends, but are increased by 11 bp when the test bend containing three A tracts is used. The *Pvu*II (P) and *Nco*I (N) sites used to generate probes with different lengths of flanking sequences are indicated. Although the fragments are drawn approximately to scale, they represent average conformations in dynamic populations with broad distributions of bend angles.

premise has not been directly tested, the close correspondence between the electrophoretic mobility variations of DNA fragments containing intrinsic bends and the protein−DNA complexes under a variety of conditions supports this assumption.

The direction of protein-induced DNA bending was defined at the center of the AP-1 site relative to the direction of intrinsic DNA bending defined at the center of the A tract. The choice of the position at which bend direction is defined is arbitrary and does not necessarily correspond to a locus of bending or the center of the bend for either the A tract or the bend induced by Fos and Jun. The DNA bend direction was calculated from the difference between the separations that resulted in maximum mobility for the protein−DNA complexes and for fragments containing an intrinsic A tract bend (S_{T_S}). This phase shift reflects the difference between the directions of bending induced by the complex and by the A tract. The difference in bend direction was expressed as a torsion angle based on the 10.4 bp/turn periodicity (P) determined for the sequence between the two bends. Positive torsion angles were defined in a clockwise direction when the DNA is viewed from the side opposite to the reference bend. DNA bending by phased A tracts has been shown to be directed toward the minor groove at a position displaced by a little less than half a base pair toward the 3′ end of the A tract (Crothers & Drak, 1992). Although a displacement of 0.3 bp would be consistent with DNA bending by Jun homodimers in a direction parallel to the major groove−minor groove axis at the center of the AP-1 site based on the experiments presented here, the absolute direction of DNA bending was not calculated since the direction of A tract bending has not been independently determined at this

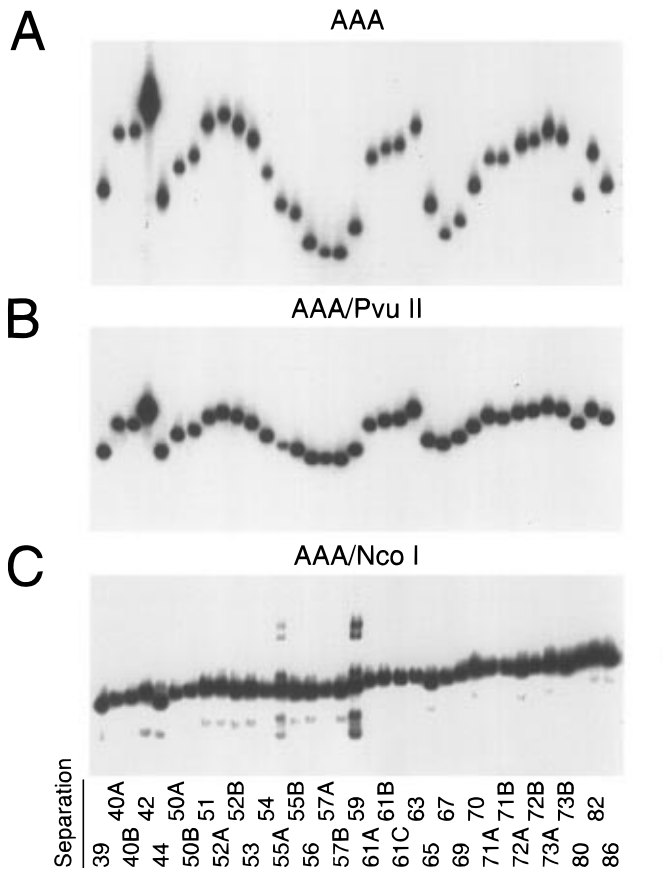


FIGURE 2: Effects of the separation between two intrinsic bends and the length of flanking sequences on the mobility variation of fragments containing two equivalent bends. The mobilities of DNA fragments containing two bends, each consisting of three phased A tracts, were analyzed by electrophoresis in a 5% polyacrylamide gel at RT. The separations between the centers of the bends are indicated at the bottom of the figure. In cases where several fragments with the same separation, but different sequences separating the intrinsic bends were analyzed, they are differentiated by letters (Kerppola, 1996). The lengths of the flanking sequences were 151 bp (AAA), 58 bp (AAA/*Pvu*II), and 25 bp (AAA/*Nco*I). The time of electrophoresis was adjusted such that the average mobility of each set of fragments was comparable. Only part of the gels is shown to conserve space. The upper/lower edges of the images were 100/170 mm (A), 105/165 mm (B), and 90/150 mm (C) from the wells.

level of accuracy. Thus, the directions of bending reported represent the differences in bend directions between the complexes and the A tract when they are centered at the same position.

RESULTS

Phasing analysis is based on the phase dependent variation in the electrophoretic mobilities of DNA fragments containing two bends separated by spacers of different lengths (Figure 1). When the two bends are placed on the fragment such that they cooperate to increase the overall extent of bending, they are said to be in phase, whereas when they counteract each other to reduce the net DNA bend they are said to be out of phase. DNA bends that are in phase are presumed to reduce the electrophoretic mobility, relative to the effect of either of the bends alone, whereas DNA bends that are out of phase are presumed to increase the electrophoretic mobility. Several experimental parameters, including the separation between the DNA bends, the length of flanking sequences, and the acrylamide concentration used

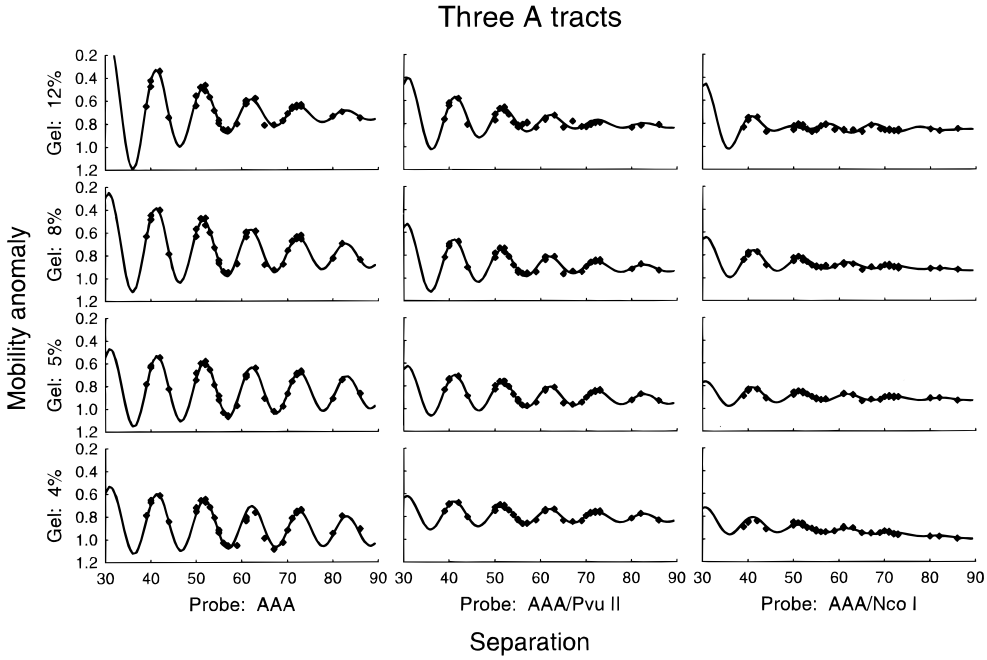


FIGURE 3: Multifactorial phasing analysis of an intrinsic bend containing three phased A tracts. The mobility anomalies of DNA fragments containing two intrinsic bends, each consisting of three phased A tracts, were plotted as a function of the separation between the centers of the bends. The mobility anomaly is defined here as the ratio between the actual length of the fragment and its apparent length based on its electrophoretic mobility, and was approximately proportional to the relative mobility used to plot complex mobilities in Figure 6. Thus, fragments with a smaller mobility anomaly migrated slower relative to their size than fragments with a larger mobility anomaly. Some fragments with out of phase bends were found to migrate slightly faster than standards of the same length. This may indicate that the standards contain small DNA bends, or that differences in the base composition or the ends of the fragments cause slight differences in electrophoretic mobility. The mobilities of fragments containing 151 bp (AAA, left column), 58 bp (AAA/*Pvu*II, middle column), and 25 bp (AAA/*Nco*I, right column) flanking sequences were analyzed by electrophoresis at 4 °C in gels prepared using 12%, 8%, 5%, and 4% acrylamide solutions (rows top to bottom). The best fit of a damped phasing function to each set of data is shown.

to prepare the gel, can influence the relative electrophoretic mobilities of DNA fragments containing in phase and out of phase bends. These parameters were systematically varied, and their effects on the electrophoretic mobility variation caused by intrinsic and protein-induced DNA bends were compared. This approach is designated multifactorial phasing analysis.

Multifactorial Phasing Analysis of Intrinsic DNA Bending. The quantitative interpretation of results from phasing analysis rests on the assumption that the effect of two DNA bends on the electrophoretic mobility of a DNA fragment is equivalent to the effect of a single contiguous DNA bend, corresponding to the sum of the bends when they are in phase and the difference between the bends when they are out of phase. To test this assumption, and to develop a general method for determining whether the phase-dependent mobility variation of protein−DNA complexes is caused by DNA bending, the mobilities of fragments containing two equivalent intrinsic bends in different spatial arrangements (Figure 1: AAA) were determined under a variety of electrophoresis conditions (Figure 2). The separation between the centers of the bends was varied from 39 to 86 bp. The bends were tested on DNA fragments of different lengths containing 151, 58, and 25 bp flanking sequences. The mobilities of the fragments were examined in gels prepared using 4%, 5%, 8%, and 12% acrylamide solutions. A subset of the fragments was analyzed by gel electrophoresis both at room temperature as well as at 4 °C.

The mobilities of DNA fragments containing two intrinsic bends were compared with those of size standards composed of mixed sequence DNA. The ratio between the known length of the fragments based on DNA sequence and their apparent length based on electrophoretic mobility was plotted as a function of the separation between the centers of the intrinsic bends (Figure 3). These mobility anomalies were fitted to a harmonic oscillation function with exponential decay, called the damped phasing function. The period of this function was 10.4 bp under most conditions, reflecting the period of the DNA sequences inserted between the intrinsic bends. Thus, intrinsic bends cooperate in their effects on electrophoretic mobility when placed integral turns apart on DNA fragments with long flanking sequences.

The phase-dependent mobility variation of DNA fragments containing two intrinsic bends was markedly affected by the separation between the bends, by the length of flanking sequences, and also by the acrylamide concentration. When the separation between the bends was increased, the amplitude of the cyclic variation in their mobilities decreased. Similarly, truncation of flanking sequences resulted in a decrease in the mobility variation. The combined effect of increasing the separation between the bends and reducing the length of flanking sequences was the complete loss of the mobility variation. It is therefore clear that the previously reported failure to detect a variation in the mobilities of complexes formed by Fos and Jun on phase-sensitive detection probes with a separation of 65 bp between the AP-1 site and the reference bend and 30 bp flanking sequences (Sitlani & Crothers, 1996) was due to the geometry of the probes, and does not indicate a lack of DNA bending.

Comparison of the mobilities of fragments containing two bends (Figure 3) with those of fragments containing a single contiguous DNA bend (Figure 4) demonstrated that the assumptions of quantitative phasing analysis are valid only for DNA fragments in which the two bends are closely

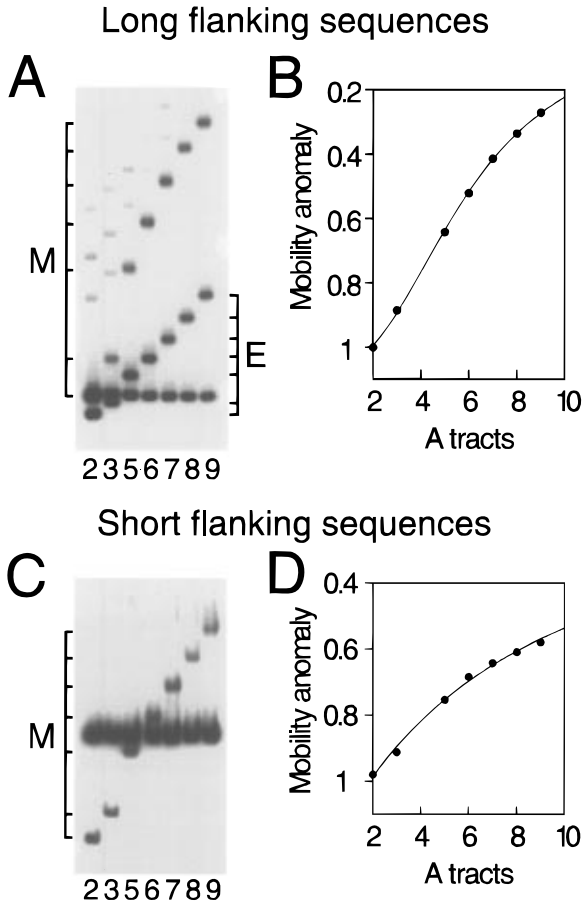


FIGURE 4: Effects of the length of flanking sequences on the electrophoretic mobilities of DNA fragments containing DNA bends of different magnitudes. The mobilities of DNA fragments containing intrinsic bends consisting of different numbers of phased A tracts were analyzed by electrophoresis in 5% polyacrylamide gels (A and C). The number of A tracts in each fragment is indicated at the bottom of each figure. DNA fragments with long flanking sequences (A and B) contained 160 bp on each side of the A tracts whereas DNA fragments with short flanking sequences (C and D) contained 40 bp on each side. To show the effect of the position of the intrinsic bend on electrophoretic mobility, fragments containing the bends in the middle (M) were mixed with fragments containing the bends near one end (E). Fragments lacking phased A tracts were used as internal standards in each experiment. The mobility anomalies of the fragments were calculated from the ratio between the length of the fragment and its apparent length based on its electrophoretic mobility relative to DNA size standards.

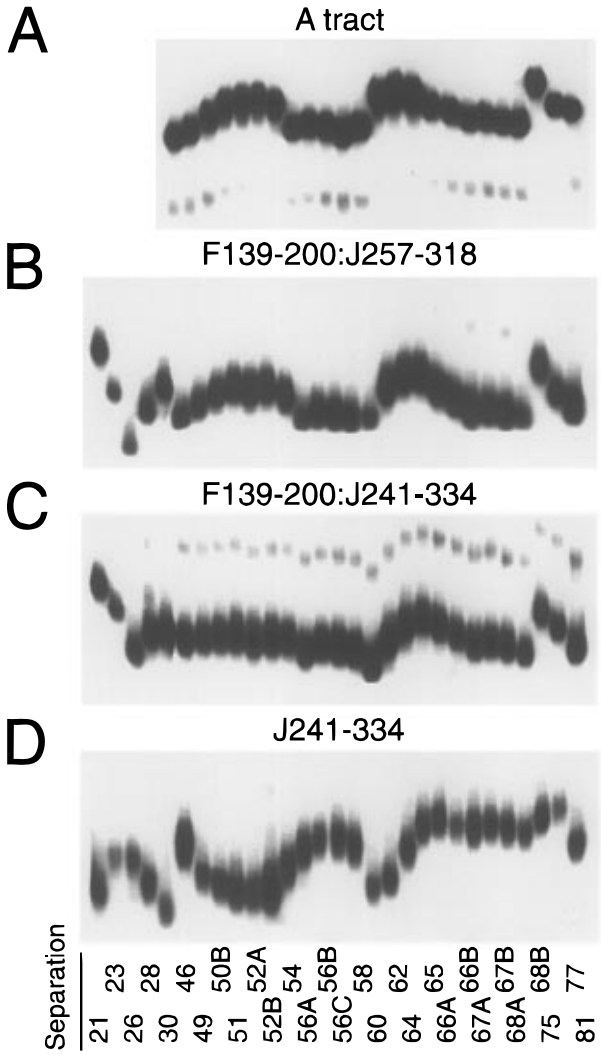


FIGURE 5: Comparison of the electrophoretic mobility variations induced by the binding of different complexes formed by truncated Fos and Jun with that caused by an intrinsic A tract test bend. The mobilities of DNA fragments containing an intrinsic bend (A) or complexes formed by Fos139-200:Jun257-318 (B), Fos139-200:Jun241-334 (C), and Jun241-334 (D) at different distances from a reference bend were analyzed in a 4% polyacrylamide gel at 4 °C. The separation between the center of the reference bend and the A tract test bend or the AP-1 site is indicated at the bottom of the figure. All fragments contained 150 bp flanking sequences. Only part of the gels is shown to conserve space. The upper/lower edges of the images were 80/125 mm (A) and 140/185 mm (B, C, and D) from the wells.

spaced and have long flanking sequences. Two closely spaced bends that are in phase caused a mobility anomaly similar to that caused by a bend representing the sum of the bends on DNA fragments with long flanking sequences. However, when the separation between the bends was increased, their effect on electrophoretic mobility decreased. Two bends that are in phase, but separated by a long spacer, therefore do not cooperate in their effects on electrophoretic mobility to the same extent as two closely juxtaposed bends. The electrophoretic mobilities of DNA fragments containing two out of phase bends of the same magnitude separated by a short spacer were similar to those of fragments of the same size lacking phased A tracts. In contrast, fragments with out of phase bends separated by long spacers migrated slower than predicted by their size. This is reflected in a decrease in the mobility anomaly with increasing separation between out of phase bends, particularly at higher acrylamide concentrations (Figure 3). Thus, two equivalent bends that are placed out of phase relative to one another do not cancel the effects of each other on electrophoretic mobility when separated by a long spacer. Consequently, quantitative phasing analysis should be performed using DNA fragments with a short separation between the reference and test bends and long flanking sequences.

The effects of changes in the separation between the bends, the length of flanking sequences, and the acrylamide concentration on the electrophoretic mobilities of DNA fragments with intrinsic A tract bends were interdependent. As expected based on the electrophoretic mobilities of DNA fragments containing a single DNA bend (Thompson & Landy, 1988; Zinkel & Crothers, 1990), the mobility variation of DNA fragments with closely spaced bends increased with increasing acrylamide concentration. Surprisingly, the mobility variation of DNA fragments with a long

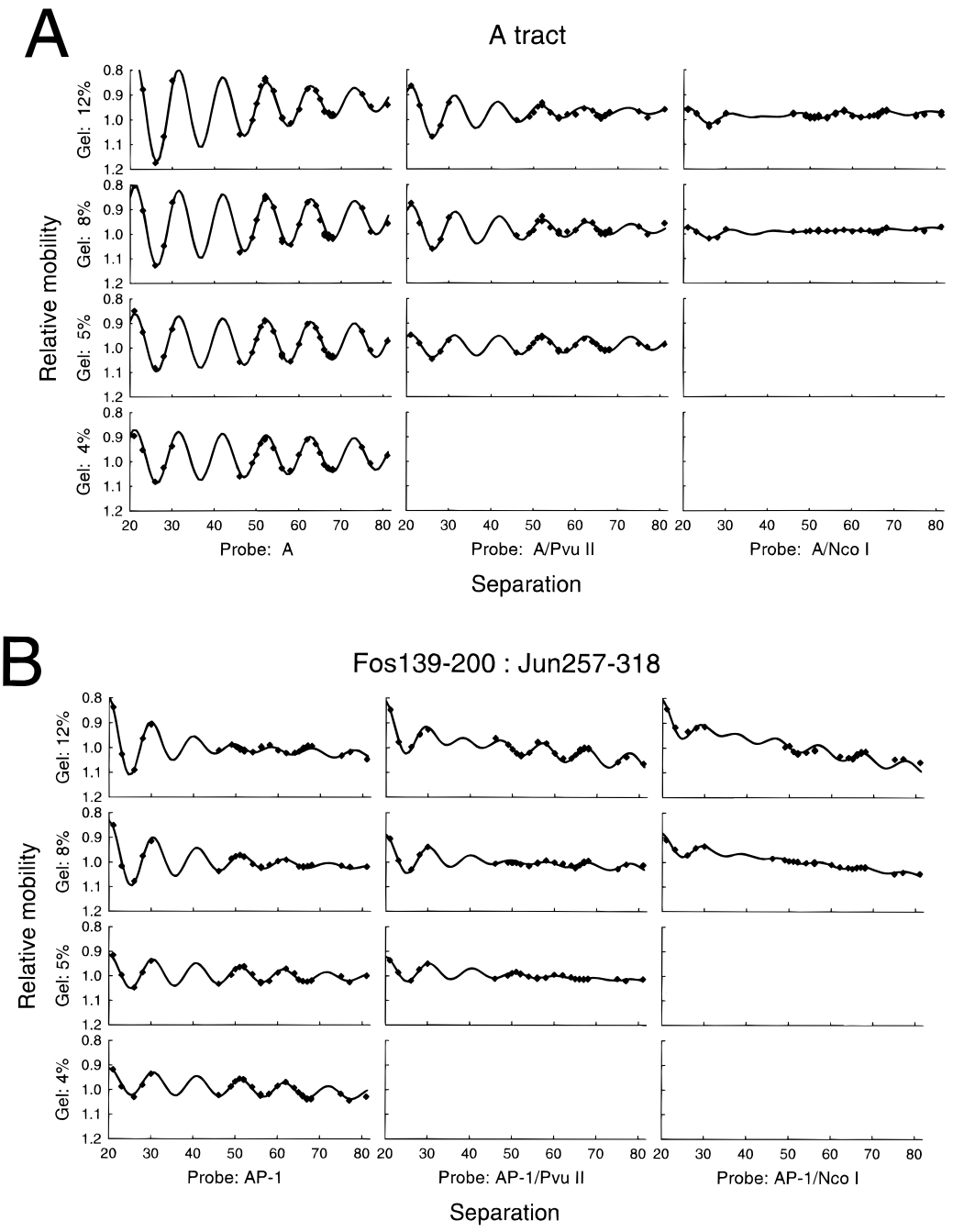
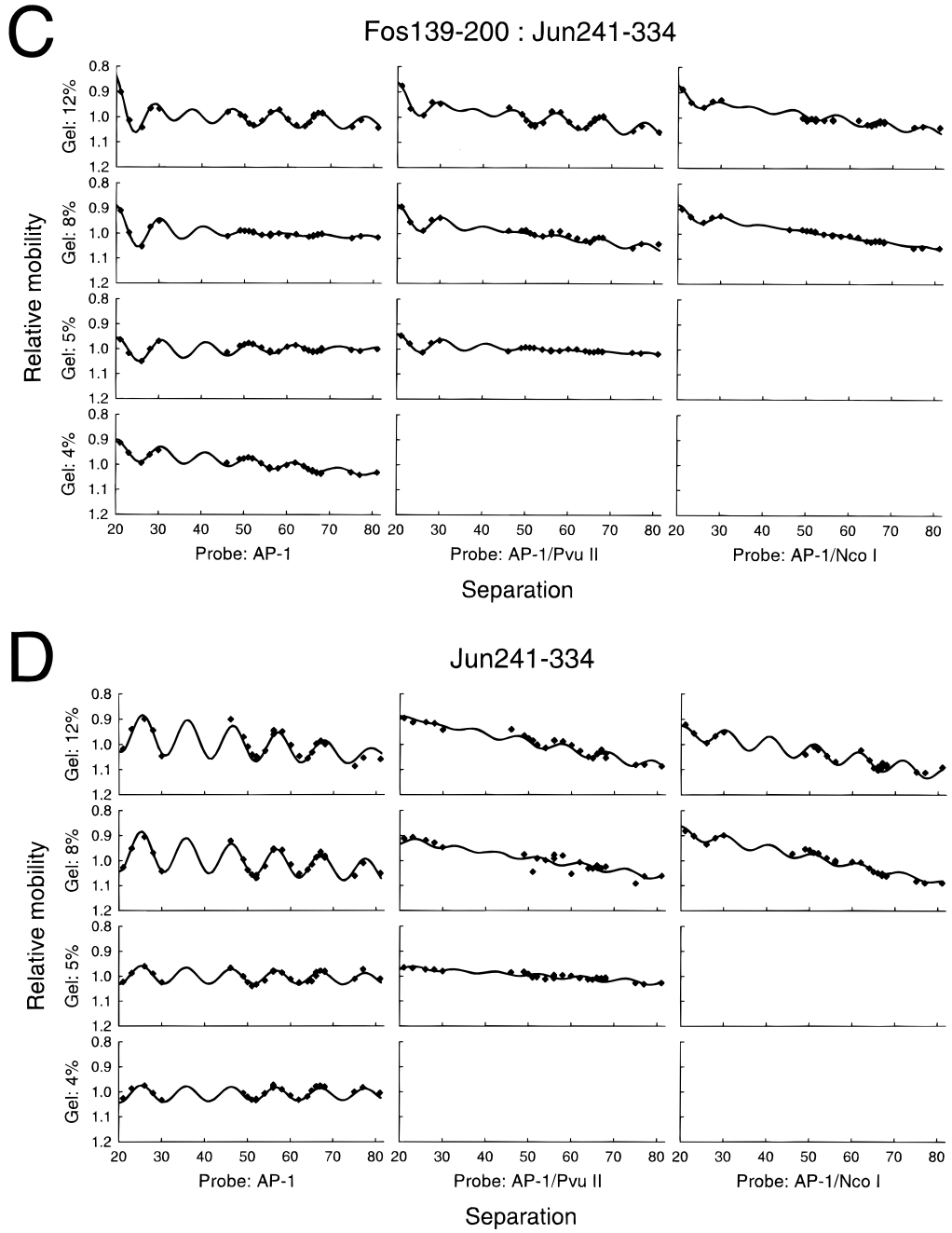


FIGURE 6: Comparison of an intrinsic bend and complexes formed by truncated Fos and Jun by multifactorial phasing analysis. The mobilities of DNA fragments containing an intrinsic A tract bend (A) and complexes formed by Fos139−200:Jun257−318 (B), Fos139−200:Jun241−334 (C), and Jun241−334 (D) were normalized for differences in the mobilities of the AP-1 site probes alone, and were plotted as a function of the separation between the centers of the reference bend and the A tract test bend or the AP-1 site. The mobilities of each set

separation between the bends decreased with increasing acrylamide concentration (Figure 3). This decrease is due to the greater reduction in the mobility of fragments containing out of phase bends at high acrylamide concentrations. The differential retardation of the mobilities of fragments containing widely separated out of phase bends at high acrylamide concentrations was observed for all of the DNA bends examined here.

Effects of Bend Magnitude on DNA Fragment Mobility in Multifactorial Phasing Analysis. The electrophoretic mobility of DNA fragments containing a single contiguous bend is a function of the magnitude of the DNA bend angle (Thompson & Landy, 1988; Zinkel & Crothers, 1990) (Figure 4). To investigate the effect of bend magnitude on multifactorial phasing analysis, the mobilities of DNA fragments containing a single A tract test bend at different distances from the reference bend (Figure 1: A), were determined (Figure 5A). The amplitude of the mobility variation for DNA fragments containing a single A tract test bend was smaller than that of fragments containing a test bend with three A tracts under all conditions examined (Figure 6A). The ratio between these amplitudes in gels with different acrylamide concentrations was similar for DNA fragments with a short separation between the bends and long flanking sequences. Differences in acrylamide concentration therefore had comparable effects on the mobility variation caused by intrinsic bends of different magnitudes on DNA fragments that satisfy the assumptions of phasing analysis.



of complexes were determined on fragments containing 151 bp (A or AP-1, left columns), 58 bp (A or AP-1/*Pvu*II, middle columns), and 25 bp (A or AP-1/*Nco*I: right columns) flanking sequences in gels prepared using 12%, 8%, 5%, and 4% acrylamide solutions (rows top to bottom). The mobilities of fragments with short flanking sequences were not analyzed at the lowest acrylamide concentrations because of the low mobility variations under these conditions. The best fit of a damped phasing functions to each set of mobilities is shown.

The phase of the mobility variation of these fragments, reflecting the direction of DNA bending, was also constant under different electrophoresis conditions. Thus, the effect of differences in acrylamide concentration on the electrophoretic mobility variation of DNA fragments with short separations and long flanking sequences can be used as a criterion to determine whether the phase-dependent mobility variation induced by protein binding is caused by DNA bending. Furthermore, the amplitude and phase of the mobility variation of these fragments can be used to calculate the magnitude and direction of DNA bending.

The mobility variation of fragments containing intrinsic DNA bends was reduced by an increase in the separation between the bends. Under most conditions, the effect of increasing the separation between the bends was distinct for intrinsic bends of different magnitudes. However, at low acrylamide concentrations, an increase in the separation between the reference and test bends had similar effects on the mobility variations of fragments containing intrinsic A tract bends of different magnitudes with long flanking sequences (Table 1). The effect of the separation between bends on the electrophoretic mobility variation at low acrylamide concentrations can therefore provide an additional criterion to determine whether the phase-dependent variation in the electrophoretic mobilities of protein complexes is due to DNA bending.

The effect of reducing the length of sequences flanking intrinsic bends separated by long spacers was unexpected. At high acrylamide concentrations, the phase of the mobility variation of such fragments was reversed. This reversal of

Table 1: Analysis of DNA Bending under Different Electrophoresis Conditions

		acrylamide concentration			
		12%	8%	5%	4%
three A tracts	amplitude (A_{31_S})[a]	1.91	1.31	0.91	0.73
	half-separation ($S_{1/2}$)[b]	14	25	41	43
A tract	amplitude (A_{21_S})	0.48	0.36	0.25	0.22
	half-separation ($S_{1/2}$)	20	35	46	46
F139−200: J257−318	amplitude (A_{21_S})	0.29	0.21	0.15	0.13
	bend angle[c]	11	11	11	11
	phase shift[d]	−0.2	−0.2	−0.3	−0.4
	bend direction[e]	352	352	351	345
F139−200: J241−334	amplitude (A_{21_S})	0.17	0.15	0.09	0.07
	bend angle	6	7	6	6
	phase shift	−0.1	−0.1	−0.1	0.0
	bend direction	358	358	357	359
J241−334	amplitude (A_{21_S})	0.19	0.16	0.10	0.09
	bend angle	7	8	7	7
	phase shift	4.9	5.0	4.9	4.8
	bend direction	169	172	170	164
Fos−Jun	amplitude (A_{21_S})			0.16	
	bend angle			12	
	phase shift			−0.1	
	bend direction			356	
Jun	amplitude (A_{21_S})			0.22	
	bend angle			16	
	phase shift			5.1	
	bend direction			176	
Fos:J257−318	ampltidue (A_{21_S})			0.34	
	bend angle			24	
	phase shift			−0.1	
	bend direction			356	
F139−200:Jun	amplitude (A_{21_S})			0.02	
	bend angle			2	
	phase shift			2.4	
	bend direction			82	

[a] The amplitude of the variation in the relative mobilities was calculated at the separation where the two bends merge to form a contiguous bend (31 bp for AAA and 21 bp for A and AP-1). [b] The half-separation (in bp) represents the increase in the separation that results in a 50% decrease in the mobility variation. [c] The bend angle (in degrees) was calculated from the ratio between the amplitudes of the protein-induced and A tract bends. [d] The phase shift (in bp) represents the difference in phase between the protein-induced and A tract mobility variation. [e] The bend direction (in degrees) was calculated from the phase shift and represents the difference in bend direction between the protein-induced and A tract bends defined at the centers of the respective sequences.

the phase of the mobility variation resulted from the combined effects of a decrease in the mobilities of DNA fragments containing out of phase bends relative to the mobilities of fragments containing in phase bends at high acrylamide concentrations, and an increase in the relative mobilities of fragments with in phase bends upon truncation of flanking sequences. The phase of the mobility variation was reversed for DNA fragments with intrinsic bends of different magnitudes. However, the relative effects of acrylamide concentration and the length of flanking sequences were distinct for different bends. Thus, a final criterion to determine whether the phase-dependent variation in electrophoretic mobility is caused by DNA bending is reversal of the phase of the mobility variation for DNA fragments with long separations and short flanking sequences at high acrylamide concentrations.

Multifactorial Phasing Analysis of DNA Bending by Fos and Jun. The multifactorial phasing analysis approach was tested using homo- and heterodimers formed by truncated Fos and Jun proteins encompassing the bZIP dimerization and DNA binding domains. The intrinsic A tract bend was substituted by an AP-1 site (Figure 1: AP-1), and the electrophoretic mobilities of homo- and heterodimeric complexes formed by truncated Fos and Jun proteins were determined in parallel with DNA fragments containing intrinsic A tract test bends (Figure 5). The mobilities of the complexes as well as fragments containing an A tract bend were normalized for differences in the mobilities of the AP-1 site probes alone and plotted as a function of the separation between the centers of the intrinsic bend and the AP-1 site. Thus, the relative mobilities of the complexes can be compared directly to the relative mobilities of DNA fragments containing an intrinsic bend (Figure 6).

The mobility variations induced by truncated Fos and Jun at different acrylamide concentrations were proportional to those caused by an intrinsic bend on fragments that satisfy the assumptions of phasing analysis. Thus, on DNA fragments with short spacers and long flanking sequences, differences in acrylamide concentration had the same effect on the mobility variations caused by Fos−Jun complexes and intrinsic bends, consistent with the first criterion for DNA bending. The significance of the constant ratio between the mobility variations of Fos−Jun complexes and intrinsic bend fragments over the wide range of acrylamide concentrations tested is amplified by the 10-fold variation in the absolute mobilities of the complexes under these conditions. Potential artifacts caused by protein structure are highly unlikely to have the same effect as intrinsic DNA bends on the mobility variation at different acrylamide concentrations. Thus, comparison of the electrophoretic mobility variation of protein−DNA complexes at different acrylamide concentrations is a simple and powerful approach to determine if their phase-dependent mobility variation is caused by DNA bending.

An increase in the separation between the reference bend and the complexes formed by truncated Fos and Jun had distinct effects on the mobility variations of the different complexes under most conditions. However, at low acrylamide concentrations, the mobility variations caused by intrinsic bends and complexes formed by Fos and Jun on DNA fragments with long flanking sequences were proportional over the entire range of separations examined. Similarly, differences in separation had distinct effects on the mobility variation caused by intrinsic bends of different magnitudes, with the exception of intrinsic bends with long flanking sequences analyzed at low acrylamide concentrations. Thus, under these conditions, differences in the separation between the bends had identical effects on the mobility variations of both complexes formed by Fos and Jun as well as intrinsic DNA bends of different magnitudes. Therefore, all of the complexes formed by truncated Fos and Jun fulfill the second criterion for DNA bending. Consequently, under all conditions where intrinsic bends of different magnitudes had the same relative effects on the mobility variation, complexes formed by truncated Fos and Jun also induced the same relative variation in electrophoretic mobilities.

A decrease in the length of flanking sequences had distinct effects on the mobility variations caused by the various complexes formed by truncated Fos and Jun. However, for all complexes, the phase of the mobility variation was reversed at high acrylamide concentrations on DNA frag-

ments with long separations and short flanking sequences. Thus, these complexes fulfill the third criterion for DNA bending. The relative effects of changes in the length of flanking sequences on the mobilities of complexes with short separations varied among the different complexes. Likewise, differences in the length of flanking sequences had quantitatively distinct effects on the mobility variations caused by intrinsic bends of different magnitudes. In addition to bend magnitude, the effects of these parameters may be affected by differences in the geometry and dynamics of the DNA bend. Consequently, the variation in the electrophoretic mobilities of complexes formed by truncated Fos and Jun under a wide variety of circumstances is consistent with DNA bending.

Quantitation of DNA Bending by Truncated Fos and Jun through Multifactorial Phasing Analysis. The magnitudes of the bends induced by the various truncated Fos and Jun complexes were quantitated by comparison of the amplitudes of the mobility variations induced by the complexes with those caused by intrinsic A tract bends on DNA fragments with short separations and long flanking sequences (Table 1). The identical sequence of these DNA fragments with the exception of the substitution of the AP-1 site for the A tract and the parallel analysis of electrophoretic mobility allows direct comparison of the mobility variations caused by the protein−DNA complexes and the intrinsic bends. The amplitudes of the mobility variations were compared at a separation of 21 bp (A_{21_S}), the distance at which the single A tract test bend and the reference bend consisting of three A tracts merge to form a contiguous DNA bend. The magnitudes of the bends were determined independently under different electrophoresis conditions. The deduced DNA bend angles were highly consistent for each complex under all conditions examined (Table 1).

The direction of protein-induced DNA bending was determined by comparison of the phase of the mobility variation induced by the protein−DNA complex with that caused by an intrinsic A tract bend. This phase shift reflects the difference between the directions of bending induced by the complex and by the A tract. The difference in bend direction was calculated based on the 10.4 bp/turn periodicity determined for the DNA separating the bends. Since the AP-1 site and the A tract test bend were separated from the reference bend by the same sequences, the helical repeat of the sequences separating the sites in the absence of protein was the same. Protein-induced changes in the helical periodicity between the AP-1 site and the reference bend will affect the direction of DNA bending both as measured by phasing analysis as well as from the perspective of proteins bound on opposite sides of the binding site. Both restriction fragment and oligonucleotide cyclization experiments indicate that Fos and Jun do not cause significant changes in helical periodicity upon binding to the AP-1 site (Sitlani & Crothers, 1996; Kerppola, 1996). Thus, the directions of DNA bending induced by Fos and Jun are unlikely to be affected by protein-induced changes in helical pitch. The directions of DNA bending determined under different conditions were also highly consistent for each complex (Table 1).

Since the mobility variations in gels with different acrylamide concentrations were determined independently, the consistent DNA bend angles and directions determined under different conditions attest to the reliability of bend measurements based on phase dependent variation in electrophoretic mobility.

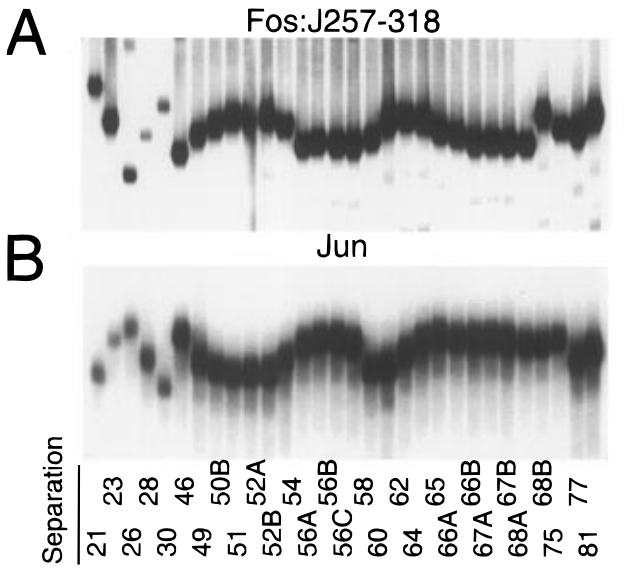


FIGURE 7: Effects of regions outside the bZIP domains of Fos and Jun on the electrophoretic mobility variation. The mobilities of complexes formed by full length Fos−truncated Jun heterodimers (A) and full-length Jun homodimers (B) were analyzed by electrophoresis in a 5% polyacrylamide gel at 4 °C. The separations between the AP-1 site and the intrinsic bend are indicated at the bottom of the figure. All fragments contained 151 bp flanking sequences. Only part of the gels is shown to conserve space. The upper/lower edges of the images were 60/110 mm (A) and 45/95 mm (B) from the wells.

The directions of DNA bending induced by complexes formed by Fos and Jun were rotated by 350° (F139−200:J257−318), 358° (F139−200:J241−334), and 169° (J241−334) clockwise relative to the direction of A tract bending when viewed from the side opposite to the reference bend. DNA bending by phased A tracts is directed toward the minor groove at a position shifted a little less than one-half base pair 3′ to the center of the A tract (Crothers & Drak, 1992). This shift agrees remarkably well with the 11° counterclockwise rotation of the direction of bending by Jun homodimers relative to the minor groove−major groove axis at the center of the AP-1 site. It is therefore likely that truncated Fos−Jun (F139−200:J257−318) heterodimers as well as Jun (J241−334) homodimers bend DNA in opposite directions parallel to the major groove−minor groove axis at the center of the AP-1 site whereas the direction of DNA bending by phased A tracts is rotated clockwise by approximately 11° relative to this axis at the center of the A tract.

Influence of Regions Outside the bZIP Domains on DNA Bending by Fos and Jun. The variation in the electrophoretic mobilities of complexes formed by full length Fos and Jun on phasing analysis probes is larger than that of complexes formed by the truncated proteins (Kerppola & Curran, 1991a,b). To investigate if this greater mobility variation is caused by increased DNA bending, the electrophoretic mobilities of complexes formed by homo- and heterodimers composed of various combinations of full-length and truncated proteins at different distances from a reference bend were determined (Figure 7). Their mobility variations were analyzed at low acrylamide concentrations on DNA fragments with long flanking sequences, conditions under which differences in separation had comparable effects on the

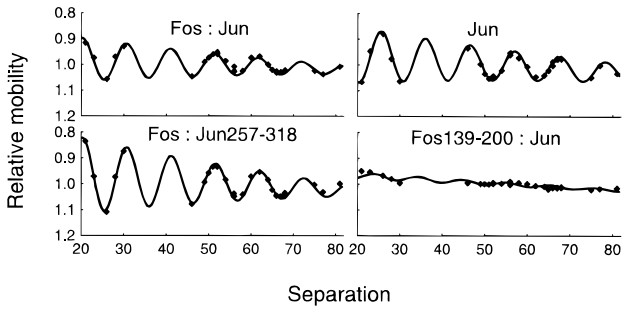


FIGURE 8: Effect of the distance between the reference bend and complexes formed by full-length and truncated Fos and Jun on the electrophoretic mobility variation. The electrophoretic mobilities of complexes formed by the proteins indicated above each graph were normalized for differences in probe mobilities, and were plotted as a function of the separation between the centers of the AP-1 site and the reference bend. All complexes were analyzed by electrophoresis in a 5% polyacrylamide gel at 4 °C and contained 151 bp flanking sequences. The best fit of a damped phasing function to each set of mobilities is shown.

mobility variations caused by intrinsic bends of different magnitudes as well as truncated Fos and Jun complexes. The full-length Fos−Jun heterodimers and Jun homodimers caused a larger mobility variation than the truncated proteins. Heterodimers containing full-length Fos and truncated Jun caused a larger variation in complex mobilities than heterodimers containing either both full-length or both truncated proteins regardless of spacer length. In contrast, heterodimers containing full-length Jun and truncated Fos caused virtually no mobility variation on DNA fragments with either short or long spacers. These results are consistent with the model that Fos and Jun bend DNA in opposite directions, that the full-length proteins induce larger bends than the truncated proteins, and that the two subunits of the heterodimer induce independent DNA bends (Kerppola & Curran, 1991b, 1997). The effect of differences in spacing on the mobility variation of these complexes was similar to that observed for fragments containing intrinsic A tract bends as well as complexes formed by truncated Fos and Jun (Figure 8). These complexes therefore fulfill the second criterion for DNA bending. Although DNA bending by these complexes has not been examined based on the other criteria, independent results indicate that they induce DNA bending (Kerppola & Curran, 1997). Consequently, DNA bending by a variety of complexes formed by Fos and Jun proteins has been confirmed by multifactorial phasing analysis, demonstrating that this method provides a general approach to determine whether the phase-dependent mobility variation induced by protein binding is due to DNA bending.

DISCUSSION

The influence of molecular shape on the electrophoretic mobility of DNA fragments provides a powerful approach for the analysis of DNA bending. However, many factors in addition to DNA bending affect the shape of a protein−DNA complex, making it necessary to distinguish between effects of DNA bending and other influences. These effects cannot be distinguished by circular permutation analysis, and many protein complexes that do not induce DNA bending cause a mobility variation in circular permutation assays (Gartenberg et al., 1990; Kerppola & Curran, 1991b, 1993, Kuprash et al., 1995). Phasing analysis was proposed to discern the effect of DNA bending on electrophoretic mobility independent of other factors (Kerppola & Curran, 1991a,b). However, the validity of results from phasing analysis has been questioned based on the failure to observe a mobility variation in phase-sensitive detection assays or a change in the kinetics of ligase-catalyzed cyclization for several complexes that cause a mobility variation in phasing analysis (Sitlani & Crothers, 1996; McCormick et al., 1996). Furthermore, many proteins that bend DNA in phasing analysis experiments do not cause bending in crystals (Ferre-D'Amare et al., 1993, 1994; Glover & Harrison, 1995; Houbaviy et al., 1996). It was therefore necessary to develop improved assays for protein-induced DNA bending to resolve the contradictions among these results.

The multifactorial phasing analysis approach presented here provides a general method for determination of whether the phase-dependent mobility variation of a protein−DNA complex is caused by DNA bending. Three separate criteria are identified to define a rigorous standard for determination of whether a complex causes DNA bending. These criteria were selected based on the electrophoretic mobility variation caused by intrinsic bends of different magnitudes. They were also designed to preclude erroneous identification of DNA bending complexes. Thus, failure to meet these criteria cannot be considered proof that a complex does not induce DNA bending. It may, however, indicate that factors other than DNA bending contribute to the phase-dependent mobility variation of a complex.

Multifactorial phasing analysis was used to examine DNA bending by several complexes formed by truncated Fos and Jun proteins. All of the criteria were fulfilled by each complex, demonstrating that these complexes induce DNA bending. Complexes formed by the full-length Fos and Jun proteins were also examined under a subset of the conditions, and were found to satisfy the applicable criteria. It was not possible to examine DNA bending by full-length Fos and Jun under all conditions of multifactorial phasing analysis since the complexes did not migrate sufficiently far into the gel at high acrylamide concentrations. Heterodimers formed by truncated Jun and full-length Fos that induce no net DNA bending in phasing analysis (Kerppola & Curran, 1991b) also did not cause an electrophoretic mobility variation under any of the conditions of multifactorial phasing analysis tested. Thus, these complexes did not influence the phase-dependent mobility variation through mechanisms other than DNA bending. Indeed, although the methods presented here provide a means for identification of hypothetical complexes that induce a phase-dependent mobility variation by mechanisms other than DNA bending, no such complex has yet been identified. Therefore, these data also strengthen the premise that phasing analysis is a reliable method for identification and quantitation of protein-induced DNA bends.

Quantitation of DNA bending by phasing analysis is based on the assumption that two in phase bends have the same effect on electrophoretic mobility as a single bend corresponding to the sum of the bends whereas two out of phase bends have the same effects as a bend corresponding to the difference between the bends. This premise was found to be valid for closely spaced intrinsic bends with long flanking sequences. Thus, phasing analysis should be performed using DNA fragments with a short separation between the bends and long flanking sequences. Multifactorial phasing analysis provides several independent estimates of the

magnitude and direction of DNA bending for each complex. These estimates were consistent for all of the complexes examined. The absolute magnitudes and directions of DNA bending determined on the basis of multifactorial phasing analysis differ slightly from previous estimates (Kerppola & Curran, 1991b). These differences were mainly due to the more accurate calibration of the relationship between DNA bending and electrophoretic mobility variation derived from multifactorial phasing analysis.

The finding that the mobility variation decreased with increasing separation between the bends provides an explanation for a systematic discrepancy between the electrophoretic mobilities of Fos and Jun complexes, and the best fit of the phasing function. For virtually all complexes examined previously (Kerppola & Curran, 1991a,b, 1997; Leonard et al., 1997; Rajaram & Kerppola, 1997), the mobility anomaly of the complex with the shortest separation is larger whereas the mobility anomaly of the complex with the longest separation is smaller than predicted by the undamped phasing function. Thus, the decrease in the mobility variation with increasing separation between the bends appears to be universal for complexes formed by all derivatives of Fos and Jun, consistent with DNA bending by these complexes.

The mobilities of fragments containing widely separated out of phase bends were reduced at high acrylamide concentrations to a greater extent than the mobilities of fragments containing widely separated in phase bends. This differential retardation of the migration of fragments with out of phase bends was observed both for intrinsic A tract bends as well as for complexes formed by truncated Fos and Jun. In the case of DNA fragments with long separations between the bends and short flanking sequences, it resulted in reversal of the phase of the mobility variation. Neither the selective retardation of fragments with out of phase bends nor the reversal of the phase of the mobility variation was anticipated by prior theories of the migration of bent DNA fragments during polyacrylamide gel electrophoresis. The anomalous electrophoretic mobility of bent DNA fragments was originally explained based on the shorter end-to-end distance of such fragments (Wu & Crothers, 1984; Thompson & Landy, 1988). However, the conformation of the fragments during gel electrophoresis is distorted by passage through the polyacrylamide matrix. Thus, the end-to-end distance in solution is not an appropriate measure of the influence of a DNA bend on electrophoretic mobility (Hagerman, 1996). Indeed, the influence of the separation between the bends and the length of flanking sequences on the electrophoretic mobility variation cannot be explained based on differences in end-to-end distance. It is therefore likely that the effect of a DNA bend on electrophoretic mobility is due to the need to deform the DNA helix or the polyacrylamide matrix in order for the DNA fragment to pass through the pores of the gel (Levene & Zimm, 1989).

A DNA fragment is thought to move through the gel following a path determined by the electric field-biased random diffusion of the leading segment (deGennes, 1971, 1979; Doi & Edwards, 1978). The pores of the gel matrix encompassing this path define a flexible tube through which the DNA helix must pass. The migration of the DNA fragment is counteracted by a friction force resulting from interactions between the DNA helix and the gel matrix. DNA bending reduces the electrophoretic mobility by increasing the effective diameter of the DNA helix, resulting in increased friction. Two closely spaced bends increase the effective diameter of the DNA helix when they are in phase, but reduce the diameter, relative to a fragment containing only one bend, when they are out of phase. When the separation between the bends was increased, their influence on electrophoretic mobility was affected to a lesser extent by their phasing. The separation required to reduce the interaction between the bends by half depended on the acrylamide concentration (Table 1). At higher acrylamide concentrations, a shorter separation reduced the interaction between the bends, suggesting that the smaller pore size in such gels resulted in a narrower tube in which the effective diameter of the helix was determined over a shorter segment of DNA.

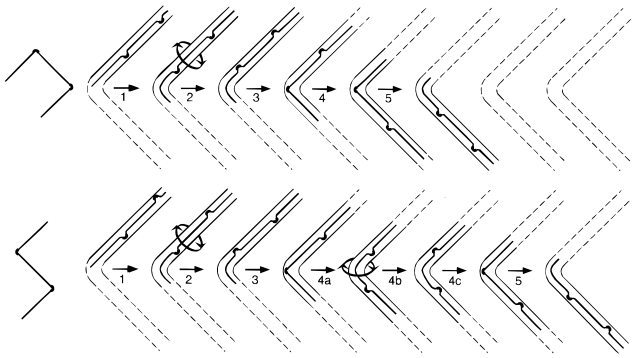


FIGURE 9: Model for the migration of DNA fragments containing two bends separated by long spacers during gel electrophoresis. The upper sequence shows the migration of a fragment containing two widely separated bends in phase whereas the lower sequence shows migration of a fragment containing two out of phase bends. The average conformations of the fragments prior to migration into the gel are shown on the left. The bends in the fragments are shown as thick arcs. The distortions required for the bends to pass through the acrylamide matrix are shown to occur exclusively in the DNA helix, although it is likely that the acrylamide matrix is distorted as well. The tubes enclosing the fragments indicate the path of DNA migration defined by the diffusion of the leading segment, and are depicted to be of uniform cross section for simplicity. The dotted portion of the tube ahead and behind the DNA fragment is shown for purposes of visualizing the motion of the fragment, and is not intended to indicate that the path of migration of the fragment is constrained prior to entry of the leading segment. In step 1, the leading segment of the fragment makes a random change in direction, generating a curve in the path of the helix. In step 2, the fragment rotates to align the first bend with the direction of the curve. Following passage of the first bend through the curve in step 3, the second bend on the fragment with two in phase bends can pass through the curve in step 4 without further rotation. However, the fragment with two out of phase bends must either rotate about the helix axis as shown in steps 4a−c or distort the second bend further in order for it to pass through the curve in the path. Thus, the fragment with two in phase bends can clear the curve in step 5 before the fragment containing two out of phase bends.

The differential retardation of DNA fragments containing widely separated out of phase bends and the reversal of the phase of the mobility variation at high acrylamide concentrations indicate that DNA bends can cooperate in their effects on electrophoretic mobility through mechanisms other than their concerted influence on the effective diameter of the DNA helix. One such mechanism may involve rotation of the DNA fragment about the helix axis to facilitate migration of bent DNA segments through random curves in the path defined by the biased diffusion of the leading segment (Figure 9). The migration of fragments containing two

widely separated out of phase bends may be retarded more than the migration of fragments with in phase bends due to the need for more frequent rotation of the fragment to align the bends with curves in the path. Protein binding is predicted to impede rotation of the fragment, consistent with the more dramatic reversal of the phase dependence of the mobility variation of complexes formed by truncated Fos and Jun. Thus, two bends on a DNA fragment can influence the effects of each other on electrophoretic mobility through multiple mechanisms that display distinct phase dependence.

A change in the phase of the electrophoretic mobility variation of fragments containing two separate intrinsic bends was observed previously at high acrylamide concentrations (Drak & Crothers, 1991). This change was attributed to differential interactions of DNA fragments containing a left-hand versus a right-hand superhelical arrangement of the bends with the acrylamide strands during rotation of the fragments through the gel matrix (Drak & Crothers, 1991). In the present experiments, no difference between the mobilities of fragments with left-hand and right-hand superhelical arrangements of the bends was observed. The most dramatic change in relative mobility was observed for fragments containing two out of phase bends in a planar arrangement. Thus, although rotation of the DNA fragments is likely to contribute to their migration through the gel, the fragments examined here display no evidence for an effect of the chirality of the DNA helix on their electrophoretic mobilities.

These studies of DNA bending by Fos and Jun by multifactorial phasing analysis confirm the validity of phasing analysis as a quantitative tool for the study of protein-induced DNA bending. When probes with short spacers and long flanking sequences were used, phasing analysis produced consistent estimates of the DNA bend angle and direction for several protein−DNA complexes under a variety of gel electrophoresis conditions. Although a comprehensive theory for understanding the anomalous electrophoretic mobilities of bent DNA fragments is lacking, these studies provide a solid empirical foundation both for the appropriate application of these methods for studies of additional protein−DNA complexes as well as for the development of such a theory. Quantitative analysis of the changes in DNA structure induced by protein binding is essential for understanding the assembly of multiprotein complexes bound to separate sites on DNA. Multiple approaches, including phasing analysis, will be needed to determine the relative contributions of protein-induced DNA bending, DNA looping through protein−protein interactions and intrinsic DNA bending to the distortion of the DNA helix required for the assembly of active transcription complexes.

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REFERENCES

Bazett-Jones, D. P., Leblanc, B., Herfort, M., & Moss, T. (1994) *Science 264*, 1134−1137.

Becker, J. C., Nikroo, A., Brabletz, T., & Reisfeld, R. A. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 9727−9731.

Crothers, D. M., & Drak, J. (1992) *Methods Enzymol. 212*, 46−71.

deGennes, P.-G. (1971) *J. Chem. Phys. 55*, 572−579.

deGennes, P.-G. (1979) *Scaling Concepts in Polymer Physics*, Cornell University Press, Ithaca, NY.

Doi, M., & Edwards, S. F. (1978) *J. Chem. Soc., Faraday Trans. 2 74*, 1789.

Drak, J., & Crothers, D. M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3074−3078.

Erie, D. A., Yang, G., Schultz, H. C., & Bustamante, C. (1994) *Science 266*, 1562−1566.

Ferre-D'Amare, A., Prendergast, G. C., Ziff, E. B., & Burley, S. K. (1993) *Nature 363*, 38−45.

Ferre-D'Amare, A. R., Pognonec, P., Roeder, R. G., & Burley, S. K. (1994) *EMBO J. 13*, 180−189.

Fisher, D. E., Parent, L. A., & Sharp, P. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11779−11783.

Gartenberg, M. R., Ampe, C., Steitz, T. A., & Crothers, D. M. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 6034−6038.

Glover, J. N., & Harrison, S. C. (1995) *Nature 373*, 257−261.

Griffith, J. D., Makhov, A., Zawel, L., & Reinberg, D. (1995) *J. Mol. Biol. 246*, 576−584.

Hagerman, P. J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4632−4636.

Hagerman, P. J. (1985) *Biochemistry 24*, 7033−7037.

Hagerman, P. J. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 9993−9996.

Heyduk, T., & Lee, J. C. (1992) *Biochemistry 31*, 5165−5171.

Houbaviy, H. B., Usheva, A., Shenk, T., & Burley, S. K. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 13577−13582.

Kahn, J. D., & Crothers, D. M. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6343−6347.

Kerppola, T. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 10117−10122.

Kerppola, T. K., & Curran, T. (1991a) *Cell 66*, 317−326.

Kerppola, T. K., & Curran, T. (1991b) *Science 254*, 1210−1214.

Kerppola, T. K., & Curran, T. (1993) *Mol. Cell. Biol. 13*, 5479−5489.

Kerppola, T. K., & Curran, T. (1997) *EMBO J. 16*, 2907−2916.

Kuprash, D. V., Rice, N. R., & Nedospasov, S. A. (1995) *Nucleic Acids Res. 23*, 427−433.

Leonard, D. A., Rajaram, N., & Kerppola, T. K. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 4913−4918.

Levene, S. D., & Zimm, B. H. (1989) *Science 245*, 396−399.

Levene, S. D., Wu, H. M., & Crothers, D. M. (1986) *Biochemistry 25*, 3988−3995.

Love, J. J., Li, X., Case, D. A., Giese, K., Grosschedl, R., & Wright, P. E. (1995) *Nature 376*, 791−795.

Lutter, L. C., Halvorson, H. R., & Calladine, C. R. (1996) *J. Mol. Biol. 261*, 620−633.

Marini, J. C., Levene, S. D., Crothers, D. M., & Englund, P. T. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7664−7668.

McCormick, R. J., Badalian, T., & Fisher, D. E. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 14434−14439.

Natesan, S., & Gilman, M. Z. (1993) *Genes Dev. 7*, 2497−2509.

Paolella, D. N., Palmer, C. R., & Schepartz, A. (1994) *Science 264*, 1130−1133.

Rajaram, N., & Kerppola, T. K. (1997) *EMBO J. 16*, 2917−2926.

Schultz, S. C., Shields, G. C., & Steitz, T. A. (1991) *Science 253*, 1001−1007.

Sitlani, A., & Crothers, D. M. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 3248−3252.

Thompson, J. F., & Landy, A. (1988) *Nucleic Acids Res. 16*, 9687−9705.

Wechsler, D. S., & Dang, C. V. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 7635−7639.

Wu, H. M., & Crothers, D. M. (1984) *Nature 308*, 509−513.

Zinkel, S. S., & Crothers, D. M. (1987) *Nature 328*, 178−181.

Zinkel, S. S., & Crothers, D. M. (1990) *Biopolymers 29*, 29−38.

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