Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 10117–10122, September 1996
Biochemistry

Fos and Jun bend the AP-1 site: Effects of probe geometry on the detection of protein-induced DNA bending

(phasing analysis/phase-sensitive detection/basic region–leucine zipper/transcription/cyclization)

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Communicated by Brian W. Mathews, University of Oregon, Eugene, OR, May 13, 1996 (received for review March 25, 1996)

ABSTRACT **The effect of Fos and Jun binding on the structure of the AP-1 recognition site is controversial. Results from phasing analysis and phase-sensitive detection studies of DNA bending by Fos and Jun have led to opposite conclusions. The differences between these assays, the length of the spacer between two bends and the length of the sequences flanking the bends, are investigated here using intrinsic DNA bend standards. Both an increase in the spacer length as well as a decrease in the length of flanking sequences resulted in a reduction in the phase-dependent variation in electrophoretic mobilities. Probes with a wide separation between the bends and short flanking sequences, such as those used in the phase-sensitive detection studies, displayed no phase-dependent mobility variation. This shape-dependent variation in electrophoretic mobilities was reproduced by complexes formed by truncated Fos and Jun. Results from ligase-catalyzed cyclization experiments have been interpreted to indicate the absence of DNA bending in the Fos–Jun–AP-1 complex. However, truncated Fos and Jun can alter the relative rates of inter- and intramolecular ligation through mechanisms unrelated to DNA bending, confounding the interpretation of cyclization data. The analogous phase- and shape-dependence of the electrophoretic mobilities of the Fos–Jun–AP-1 complex and an intrinsic DNA bend confirm that Fos and Jun bend DNA, which may contribute to their functions in transcription regulation.**

Many transcription factors alter the structure of DNA upon binding to their recognition sequences. Elucidation of the DNA structural changes induced by transcription factor binding is important for understanding both the selective binding of promoter elements (1) and the assembly of higher order nucleoprotein complexes (2–4). The most widely used methods for the analysis of protein-induced DNA bending are based on the lower electrophoretic mobilities of bent DNA fragments. However, since the mobilities of protein–DNA complexes are affected by many factors in addition to DNA structure, the original method based on this principle, circular permutation analysis, is susceptible to artifacts caused by factors unrelated to DNA bending (5–8). To circumvent these problems and to investigate the orientation of DNA bending, alternative methods, based on the phase-dependent interaction between two DNA bends, have been developed (6, 9). In these approaches, the protein-binding site is placed in tandem with an intrinsic DNA bend and the spacing, and therefore the helical phase relationship between them is varied. When two bends are in phase, they cooperate to increase the overall extent of bending, whereas when they are out of phase, they counteract and reduce the net DNA bend. Two conceptually related, yet functionally distinct, methods for the analysis of DNA bending based on phase-dependent differences in electrophoretic mobility, have been described (Fig. 1) (6, 9). The main difference between these methods lies in the relative lengths of the spacer separating the two bends and the DNA flanking the bends. In the first method, originally called phase-sensitive detection, the spacer is long relative to the flanking segments, so that the two bends are well-separated on the probe (9). In the second method, called phasing analysis, the spacer is short and the flanking segments are long, so that the two bends are closely juxtaposed (6, 10). This subtle difference in probe design can result in different conclusions regarding DNA bending (6, 10, 11). Many other methods, including ligase-catalyzed cyclization, electron and atomic force microscopy, and x-ray crystallography, can provide information about DNA bending. In some cases, results obtained in studies using different methods appear to conflict (6, 10, 12–16). It is therefore important to consider the effects of experimental parameters on the results from the different approaches.

The effect of Fos and Jun binding on the structure of the AP-1 site has been examined using many different methods (6, 10, 11, 15, 16). Fos and Jun are members of the basic region–leucine zipper (bZIP) family of proteins whose dimerization is mediated by a leucine zipper and that bind DNA through an α-helical contact interface rich in basic residues (for review, see ref. 17). Studies using gel electrophoretic phasing analysis indicate that these complexes bend DNA, and that the orientations of DNA bending induced by Fos–Jun heterodimers and Jun homodimers are opposite (6, 17). X-ray crystallographic analysis of peptides encompassing the minimal leucine zipper dimerization and basic DNA binding domains of Fos and Jun, bound to an AP-1 site, show little DNA bending (15). Recent restriction fragment cyclization and phase-sensitive detection experiments concur with the crystallographic analysis and appear to contradict the results from the earlier phasing analysis (11). The discrepancy between these results raises questions about the effect of differences in probe design on gel electrophoretic analysis of DNA bending.

MATERIALS AND METHODS

Preparation of Probes and Protein Purification. Plasmids pTK430-55 and pTK430-57 were constructed by cloning oligonucleotides containing three phased A-tracts and a polylinker spacer (shown in Fig. 2*B*) between the *Xba*I and *Sal*I sites of pTK401-26 and pTK401-28 (10). Plasmids containing spacers of different lengths were generated by restriction digestion within the spacer followed by ligation, and their sequences were confirmed by DNA sequencing. Probes were generated by PCR amplification followed by *Pvu*II digestion. Probes containing an AP-1 site separated by spacers between 31 and 66 bp from an intrinsic bend were generated by two-step PCR using primers containing an AP-1 site and spacer sequences in the first step, and flanking primers in the second step, followed by *Pvu*II digestion. Probes containing an AP-1 site separated by spacers between 6 and 15 bp from an intrinsic bend were generated by PCR amplification using plasmids pTK401-21 through pTK401-30 as templates (10).

Probes containing in-phase and out-of-phase bends with different lengths of flanking sequences were generated by PCR

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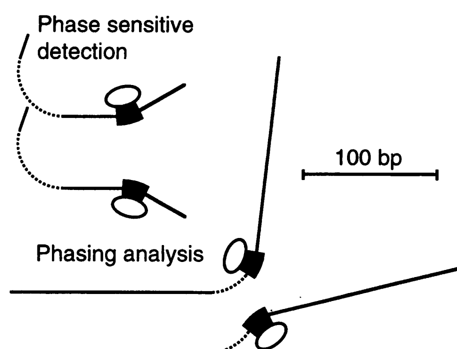

FIG. 1. Comparison of probes used in phasing analysis (bottom right) (6, 10) and phase-sensitive detection (top left) (11). The diagrams indicate the predicted differences in shape between the in-phase (upper) and out-of-phase (lower) complexes formed on the phasing analysis and phase-sensitive detection probes by a hypothetical protein (oval) that induces a 30° DNA bend upon binding its recognition site (rectangle). Note the differences in the spacing between the binding site and the intrinsic bend (dotted line) and the length of the flanking sequences on the different types of probes.

amplification with different primer pairs using plasmids A6.A2.47 and A6.A2.42 as templates (8). Smaller probes were generated by *Nco*I and *Cla*I digestion, and the correct identity of the PCR products was confirmed by *Eco*RI digestion at a site located between the two bends.

Phasing analysis plasmids containing the his3-189 site were constructed by cloning the duplex oligonucleotides XCC, XCCACCT and XCCACCTAGGT, where X=CAAAAAAA-AATGAGTCAT between the *Xba*I and *Sal*I sites of plasmids pTK401-26 and pTK401-28, and probes were prepared as above. Multimers of the his3-189 site were prepared by ligation of duplex oligonucleotides GAGX (h21), CGAGXCCAT (h26), GCTC-GAGXCCACAT (h31), and GTAGCTCGAGXCCACCTAG-GTCCAT (h42) containing asymmetric CAT/ATG overhangs.

DNA bend calibration standards were prepared by PCR amplification templated by plasmids pJT170-2 through pJT170-9, followed by digestion with *Nhe*I to generate probes containing the bend approximately in the middle or with *Bam*HI to generate probes containing the bend at the end of the fragment (18).

The truncated Fos, Jun, and Nrl proteins were expressed in *Escherichia coli* as hexahistidine fusion proteins and purified by nickel chelate affinity chromatography as described previously (10, 19–21).

Electrophoretic Mobility Analysis. Electrophoresis was performed in 29:1 acrylamide/bisacrylamide gels of the indicated concentrations in 25 mM Tris·HCl and 195 mM glycine (TG) buffer at a field strength of 1 V/cm. Proteins were added at 100 nM to 1 nM DNA probe. Electrophoresis of protein complexes was performed at 4°C to stabilize protein binding. Probe mobilities were measured by using automatic band recognition of PhosphorImager (Molecular Dynamics) scans. The apparent sizes of the probes were calculated by cubic spline interpolation between the mobilities of 123-bp ladder size standards and pBR/*Hae*III fragments smaller than 123 bp.

Ligase-Catalyzed Cyclization. Substrates for ligase-catalyzed cyclization were prepared by purification of *Bgl*I and *Ava*I digestion products of PCR amplification reactions using plasmids pTK401 and pTK401-21 through pTK401-30 as templates (10). These digestion products were between 109 and 169 bp in length, with the AP-1 site near the center, and included fragments with both integral and nonintegral numbers of helical turns. Oligonucleotide substrates were prepared by annealing duplex CCACGCATATATGACTCAT-TATACTGCACGG (*syn*) and CATATACGCGCTGACT-CAGCCGATTATCGGG (*anti*) oligonucleotides with GG/CC overhangs. These oligonucleotides are thought to differ in their preferred directions of bending as a result of the different periodicities of A+T- and G+C-rich sequences (22). The indicated concentrations of substrates and homo- or heterodimers were incubated with 50 mM Tris·HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 μg/ml BSA, and 40 units T4 DNA ligase (New England Biolabs) at 16°C. The reactions were stopped after 1 h by the addition of an equal volume of 20 mM EDTA, 0.2% SDS, 0.2 mg/ml proteinase K, 0.1 mg/ml dI·dC, 10% glycerol, bromophenol blue, and xylene cyanol. For analysis of ligation kinetics, aliquots were stopped at 5-min intervals. After 1 h incubation at 37°C, the products were separated on a polyacrylamide gel. Circular products were identified by their resistance to exonuclease digestion.

RESULTS

Effect of Spacer Length on the Mobilities of DNA Fragments Containing Two Intrinsic Bends. To investigate the effect of the differences in probe geometry between phasing analysis and phase-sensitive detection on the electrophoretic mobilities of DNA fragments, a set of model DNA structures was constructed by varying the separation between two intrinsic bends over several turns of the DNA helix. Each intrinsic bend consisted of three phased A-tracts. The mobilities of probes containing these DNA structures, ranging from shapes approximating a contiguous curve to shapes with two well-separated bends, were determined by PAGE (Fig. 2). The relative mobilities of probes containing the two bends in phase versus out of phase were dramatically different when the bends were close together, but became progressively more similar as the bends were moved further apart (Fig. 2 *A* and *C*). This reduction in the mobility difference between in-phase and out-of-phase probes with increasing separation between the bends was observed under all conditions examined.

The reduction in the mobility variation with increasing spacer length was caused primarily by a decrease in the mobility anomaly of the in-phase probes, together with a smaller increase in the mobility anomaly of the out-of-phase probes (Fig. 2*C*). Comparison with intrinsic bend standards indicated that probes containing two closely spaced bends in phase had a mobility anomaly that approached that of probes containing a single bend of the combined magnitude, whereas probes with two widely separated bends in phase had a mobility anomaly that was less than half of that of the contiguous DNA bend. In addition, probes with two out-of-phase bends that were separated by a long spacer had a mobility anomaly that was larger than that of probes with out-of-phase bends separated by a shorter spacer. Thus, bends that are separated by long spacers do not cooperate or counteract each other in their effects on electrophoretic mobility. Rather, probes that contain bends separated by long spacers have convergent mobilities that display little dependence on the phasing between the bends.

Effect of the Length of Flanking Sequences on the Mobilities of DNA Fragments Containing Two Intrinsic Bends. The second difference between phase-sensitive detection and phasing analysis is the length of the DNA segments flanking the two bends. To investigate the influence of this flanking DNA on the relative mobilities of probes containing phased bends, the length of the flanking sequences was varied on probes containing in-phase and out-of-phase bends (Fig. 3). These probes contained the reference bend used in the phase-sensitive detection studies (11), separated by 42 and 47 base pairs from a second intrinsic bend containing two phased A-tracts. Thus, the position and structural context of the second intrinsic bend on these probes are similar to that of the AP-1 site on the probes used in the phase-sensitive detection studies (Fig. 1).

The relative mobilities of probes containing in-phase and out-of-phase bends displayed a dramatic dependence on the length of the flanking sequences (Fig. 3). Probes containing 136 bp on both sides of two in-phase or out-of-phase bends migrated with markedly different mobilities, whereas probes containing 17-bp flanking sequences had identical mobilities.

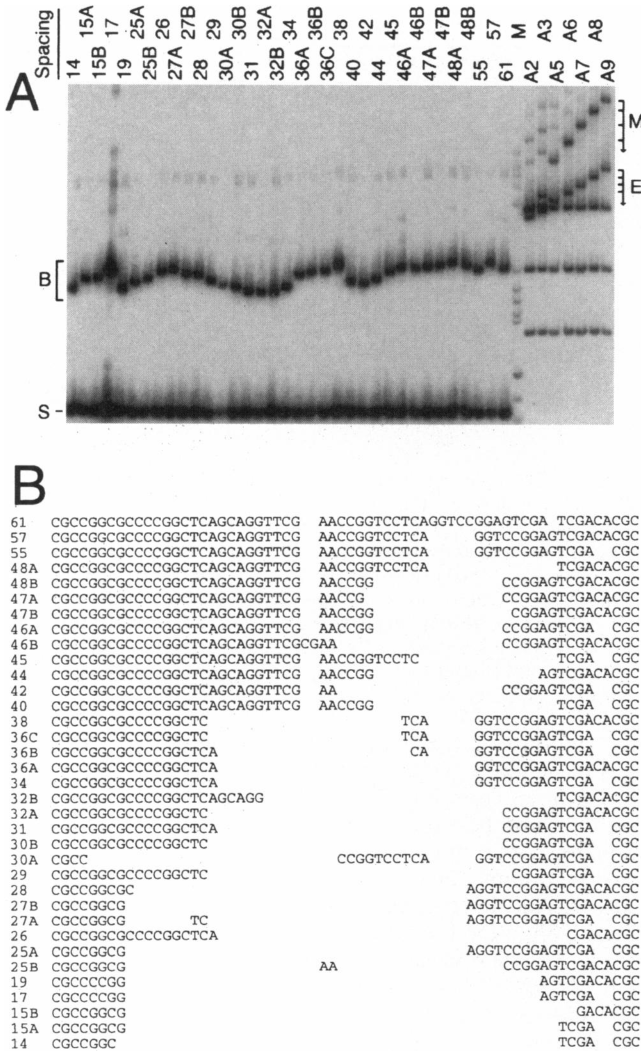

FIG. 2. Effect of spacer length on the electrophoretic mobilities of probes containing two bends. (*A*) Probes containing two intrinsic bends separated by spacers of different lengths were analyzed by electrophoresis through a 5% polyacrylamide gel. Lane designations 14–61 indicate the lengths of the spacers separating the intrinsic bends. The bands indicated by bracket B correspond to the bent probes. Band S corresponds to a straight internal control fragment. Lanes A2–A9 contain calibration standards with different numbers of phased A-tracts (18). The upper bands indicated by M contain the A-tracts at the middle of the fragment, whereas the lower bands indicated by E contain the same bend at the end of the fragment. Lane M contains pBR322 digested with *Hae*III. (*B*) Sequences of the spacers separating the intrinsic bends. Spacers of the same length are distinguished by letters. The spacers were flanked on both sides by intrinsic DNA bends consisting of three phased A-tracts (*C*) or by an intrinsic bend on one side and an AP-1 site on the other side (*D*). (*C*) Mobility anomalies of DNA fragments containing two intrinsic bends plotted as a function of the length of the spacer separating the bends. The mobility anomaly represents the ratio between the apparent size based on electrophoretic mobility and the true length of the fragments. The mobility anomalies were fitted to a damped cosine curve, which is extrapolated to 5 bp spacing to allow better comparison with the mobilities of complexes formed by truncated Fos and Jun. The brackets above the plot compare the lengths of spacers used previously in phasing analysis (6, 10) and phase-sensitive detection (11) of DNA bending by Fos and Jun. The probes contained 60-bp flanking DNA on both sides of the bends, which is shorter than the flanking segments used previously in phasing analysis, but longer than the flanking segments used in phase-sensitive detection (see Fig. 3). (*D*) Relative mobilities of complexes formed by Fos139-200–Jun257-318 (Fos′–Jun′) on probes containing spacers of different lengths separating an AP-1 site from an intrinsic bend in a 5% polyacrylamide gel. The spacers on the probes containing the AP-1 site are 5 bp longer than the corresponding spacers on the intrinsic bend probes due to the insertion of 5 bp flanking the AP-1 site. The relative complex mobilities were normalized for differences in probe mobilities.

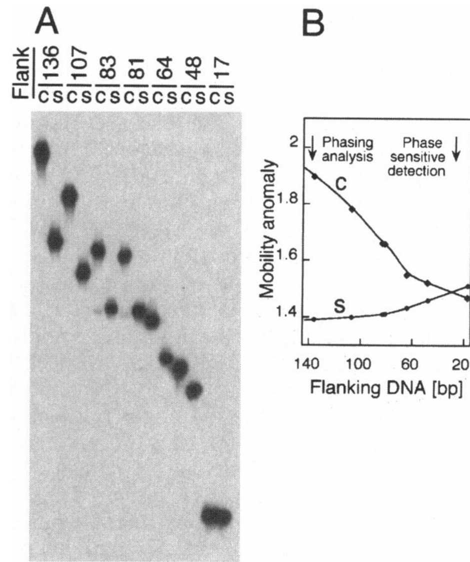

FIG. 3. Effect of the length of flanking segments on the electrophoretic mobilities of probes containing in-phase and out-of-phase bends. (*A*) Probes containing two intrinsic bends in-phase (c) or out-of-phase (s) with flanking sequences of different lengths, as indicated above the lanes were separated on an 8% polyacrylamide gel. The in-phase (c) probes contained a spacer of 42 bp, whereas the out-of-phase probes (s) contained a spacer of 47 bp. (*B*) The mobility anomalies of the fragments were plotted as a function of the length of flanking segments. Arrows above the plots compare the average lengths of the flanking segments used previously in phasing analysis (6, 10) and phase-sensitive detection (11) of DNA bending by Fos and Jun.

This lack of a mobility difference between in-phase and out-of-phase probes with short flanking segments was observed under all electrophoresis conditions examined. Thus, the mobility variation of probes containing two DNA bends with varying phasing is critically dependent on the length of the

DNA segments flanking the two bends. The mobilities of probes that most closely mimic the geometry of probes used in the phase-sensitive detection of Fos and Jun bending displayed virtually no difference in mobility. Thus, DNA bending by Fos and Jun could not be detected in the phase-sensitive detection experiments (11) because of the large separation between the intrinsic and protein-induced DNA bends and the short distance between the DNA bends and the ends of the probes.

Effect of Spacer Length on the Mobilities of Complexes Formed by Truncated Fos and Jun. The results from the experiments examining the effect of the separation between two intrinsic DNA bends on probe mobilities (Fig. 2 *A* and *C*) predicted that the mobility variation among complexes containing a protein-induced DNA bend at varying distances from an intrinsic DNA bend would decrease with increasing separation between the two bends. Additionally, the experiments examining the effect of the length of the flanking sequences (Fig. 3) predicted that the mobility variation would be smaller for complexes bound to shorter DNA probes. To examine the validity of these predictions, we constructed probes with an AP-1 site separated by spacers between 6 and 66 bp in length from an intrinsic DNA bend containing three phased A-tracts. The PCR products were digested with *Pvu*II to generate probes with 60 bp flanking the intrinsic bend and the AP-1 site. The mobilities of complexes formed by truncated Fos and Jun on these probes displayed a reduction in the mobility variation with increasing separation between the bends (Fig. 2*D*). The mobility variation was smaller than that observed previously on longer phasing analysis probes containing 150 bp flanking sequences (10). The amplitude of the mobility variation was also smaller than that observed for the intrinsic bend containing three phased A-tracts, as expected based on the smaller calculated DNA bend angle (6). The distance dependence of the mobility variation was similar for the protein complex and for the intrinsic DNA bend. This concordance supports the hypothesis that the phase-dependent mobility variation caused by the binding of truncated Fos and Jun is due to DNA bending.

DNA Bending at the his3-189 Site. Results from phase-sensitive detection studies of DNA bending by Fos and Jun at the yeast his3-189 site were interpreted to indicate the absence of DNA bending in the presence or absence of protein (11). To investigate if Fos and Jun induced bending at the his3 site by phasing analysis, probes were constructed in which this site was placed at positions close to an intrinsic DNA bend containing three phased A-tracts. Complexes formed by truncated Fos and Jun on these probes displayed a phase-dependent mobility variation, consistent with DNA bending (Fig. 4*A*). However, the mobilities of the probes varied even in the absence of protein binding. The mobility variation was reduced on probes containing shorter flanking sequences, consistent with intrinsic DNA bending. To confirm that the mobility variation was due to DNA bending at the his3-189 site, the mobilities of multimers generated by ligation of oligonucleotides containing this site were examined (Fig. 4*B*). Multimers of oligonucleotides containing an integral number of helical turns migrated with anomalously slow mobilities, whereas multimers of oligonucleotides containing a nonintegral number of helical turns migrated with normal mobilities, demonstrating that sequences at the his3-189 site caused an intrinsic DNA bend.

Effects of Truncated Fos and Jun on Ligase-Catalyzed Cyclization. Results from studies using ligase-catalyzed cyclization of restriction fragment probes have been interpreted to show that Fos and Jun do not bend DNA (11). To investigate if DNA bending by Fos and Jun would affect the cyclization of DNA fragments containing multiple tandem AP-1 sites, oligonucleotides containing an AP-1 site were ligated in the presence and absence of truncated Fos and Jun. In the presence of truncated Jun, the size distribution of circular products was shifted toward smaller circles. Addition of truncated Fos reversed this effect. To determine if this shift in the distribution of circular products was due to DNA bending, oligonucleotides that contained sequences with different preferred orientations of DNA bending were used (Fig. 5*A*). Multimers of these oligonucleotides migrated with anomalous mobilities, indicating that they were intrinsically bent. Truncated Jun homodimers promoted cyclization of oligonucleotides that favored bending in the same orientation as Jun (*syn*), but inhibited the cyclization of oligonucleotides that favored bending in the opposite orientation (*anti*). The concentration dependence of these effects was similar to that observed for Jun binding to the AP-1 site. To confirm that these effects were not the result of changes in helical twist, oligonucleotides that deviated by one base pair from an integral number of helical turns were tested with similar results. Analysis of the rates of ligation confirmed that the differences in the yields of circular and linear products reflected changes in the relative rates of intramolecular and intermolecular ligation. Substitutions in the oligonucleotide that inhibited Jun binding eliminated the effects. Truncated Fos–Jun heterodimers inhibited the cyclization of all oligonucleotides tested. These results are consistent with DNA bending by truncated Jun homodimers. However, since many other factors can affect ligation (see below), it is not possible to exclude alternative interpretations of the data.

The results from the oligonucleotide cyclization experiments contrast with the results from studies of restriction fragment cyclization (11). To investigate the difference between these approaches, restriction fragments containing AP-1 sites with and without adjacent intrinsic bends were ligated in the presence of truncated Fos and Jun. Surprisingly, both homo- and heterodimers promoted the formation of long concatemers at the expense of circular products regardless of the phasing or presence of intrinsic bends and the integral or nonintegral number of helical repeats in the probe (Fig. 5*B*). The effect was not observed with truncated Nrl homodimers that can also bind to the AP-1 site (21), indicating that it is not common to all bZIP family proteins. The stimulation of multimerization was highly sensitive to the protein concentration, but was less sensitive to the concentration of the DNA fragment. Kinetic analysis confirmed that the altered product distribution reflected a large increase in the rate of intermolecular ligation. Thus, truncated Fos and Jun can affect the relative rates of intermolecular and intramolecular ligation

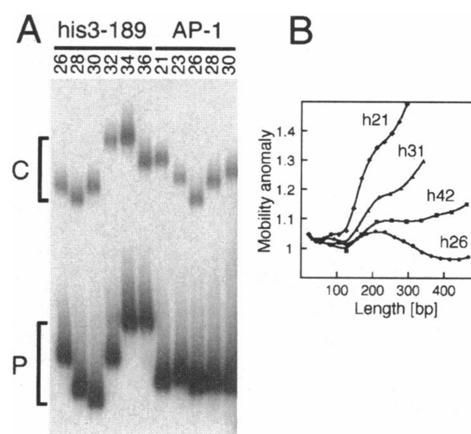

FIG. 4. Intrinsic and protein-induced DNA bending at the his3-189 site. (*A*) Phasing analysis probes with either the his3-189 or the consensus AP-1 site (6, 10) were incubated with Fos139-200–Jun257-318 heterodimers and separated on an 8% polyacrylamide gel. Note that the separation between the centers of the AP-1 sites and the intrinsic bends (shown above the lanes) differ between the two sets of probes. Bands corresponding to free probes (P) and complexes (C) are indicated by the brackets. (*B*) Mobility anomalies of oligonucleotide multimers of different lengths containing the his3-189 site separated on an 8% polyacrylamide gel.

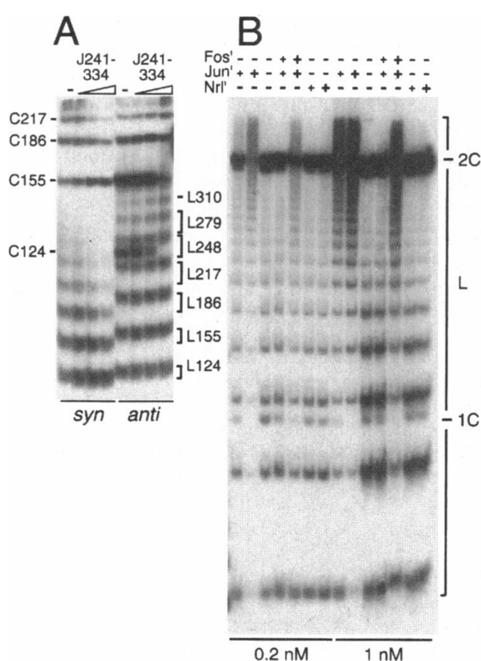

FIG. 5. Effects of Fos and Jun on ligase-catalyzed cyclization of oligonucleotide and restriction fragment substrates. (*A*) Phase-dependent stimulation of oligonucleotide cyclization by truncated Jun homodimers. Oligonucleotides that contained preexisting bends flanking the AP-1 site in the same orientation as (*syn*) or in the opposite orientation from (*anti*) the bend induced by Jun homodimers were ligated in the presence of 0, 10, 30, and 100 nM Jun241-334. The circular (C) and linear (L) reaction products were separated on by gel electrophoresis. The difference between the mobilities of the linear multimers is likely due to a slight difference in the extent of DNA bending in the two oligonucleotides. (*B*) Stimulation of restriction fragment multimerization by truncated Fos–Jun heterodimers and Jun homodimers. *Bgl*I fragments from plasmid pTK401 containing an AP-1 site in the center at 0.2 and 1 nM were ligated in the presence of 100 or 300 nM Fos139-200, Jun241-334, or Nrl116-237 as indicated above the lanes (Fos′, Jun′, and Nrl′). The monomer and dimer circles (1C and 2C) and linear multimers (L) were separated by gel electrophoresis.

through mechanisms unrelated to DNA bending, confounding the interpretation of results from cyclization experiments.

Disulfide Bond Formation Between Fos and Jun. It has been suggested that heterodimers that are bound in opposite orientations to the phase-sensitive detection probes migrate with different mobilities during gel electrophoresis (11). Complexes formed by Fos118-211:Jun225-334 on the phasing analysis probes also display two distinct mobilities under some conditions (Fig. 6). However, complexes formed by Fos139-200:Jun257-318 have a unique mobility (Fig. 4*A*). The regions that differ between these complexes include cysteine residues on the carboxyl-terminal sides of the leucine zippers of both Fos and Jun that are brought in close proximity upon dimerization. Complexes formed by proteins in which either of these cysteines was substituted by a serine migrated with unique mobilities. Thus, formation of a cysteine disulfide on the carboxyl terminal side of the leucine zipper causes a conformational change that results in altered electrophoretic mobility. If heterodimers bind to the AP-1 site in both orientations and remain bound in a fixed orientation for the duration of electrophoresis, their mobilities are not distinguishable under the conditions of these experiments.

DISCUSSION

The previous phasing analysis (6, 10) and phase-sensitive detection studies (11) of DNA bending by Fos and Jun appeared to yield contradictory results. Whereas opposite mobility patterns were observed for complexes formed by Fos–Jun heterodimers

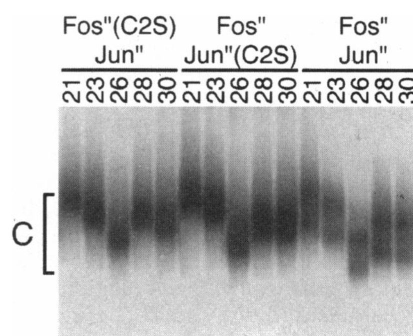

FIG. 6. Conformational variation among Fos–Jun complexes is caused by cystine disulfide formation on the carboxyl-terminal side of the leucine zipper. Complexes formed by Fos″ (Fos118-211), Jun″ (Jun225-334), Fos″ (C2S) (Fos118-211 C204S), and Jun″ (C2S) (Jun225-334 C323S) on the phasing analysis probes (10) in the presence of 1 mM DTT were separated on an 8% polyacrylamide gel.

and Jun homodimers in phasing analysis, no mobility differences were observed for either complex by phase-sensitive detection. However, the present results demonstrate that the previous studies sampled different regions of a continuum of complex mobilities that depend on both the spacing between the two bends in the complex as well as on the length of the DNA flanking the two bends. The close spacing and long flanking regions used in phasing analysis produce DNA shapes that approximate a contiguous DNA bend, providing for large mobility differences between in-phase and out-of-phase constructs. In contrast, the wide spacing and short flanking regions used in phase-sensitive detection generate more complex DNA shapes, resulting in reduced, or even undetectable, mobility differences. Some DNA bends, such as that induced by the catabolite activator protein, can be detected by phase-sensitive detection, although the mobility differences are frequently smaller than those observed in phasing analysis (9, 23). However, smaller DNA bends, such as those induced by truncated Fos and Jun or the intrinsic bend present at the his3-189 site, are not detectable in these experiments.

The shape dependence of the mobility difference between in-phase and out-of-phase probes provides an additional criterion for evaluation of the DNA bending properties of a protein complex. The qualitatively similar behavior of complexes formed by truncated Fos–Jun heterodimers and probes containing an intrinsic bend supports the interpretation that the variation in complex mobilities is caused by protein-induced DNA bending. In particular, the progressive decrease in the mobility variation with increasing separation between the bends argues against any direct interaction between the intrinsic DNA bend and the protein bound to the AP-1 site. Consequently, the lack of a variation in the mobilities of complexes formed on the phase-sensitive detection probes supports rather than contradicts the interpretation that the mobility variation in phasing analysis is caused by DNA bending.

The physical basis for the shape dependence of the mobility variation is unclear at present. The reduced mobility variation of probes containing widely separated bends may be due to the increased torsional flexibility of the longer spacer or it may reflect independent interactions of the two separate DNA bends with the gel matrix. The reduced mobility variation of probes containing shorter flanking segments is likely due to a decrease in their interactions with the gel matrix. A similar reduction was observed in the mobility anomalies of probes containing single DNA bends with shorter flanking segments (data not shown). The changes in the spacing between the bends as well as in the lengths of the flanking sequences have only a modest effect on the relative end-to-end distances of in-phase and out-of-phase probes. Thus, conventional polyanion electrophoresis theory (18, 24) cannot adequately explain the observed differences in electrophoretic mobility. It is

necessary to consider the friction forces that act on molecules of different shapes during migration through the gel matrix. Such forces have been incorporated into simulations of DNA migration during gel electrophoresis (25). However, no general theory of gel electrophoresis that considers the interactions between DNA and the gel matrix has been reported. Thus, interpretation of the data is at present limited to a qualitative level. The difference between phase-sensitive detection and phasing analysis can be most simply described as a failure of gel electrophoresis to discriminate between c-shaped and s-shaped molecules, whereas, under the same conditions, U-shaped and I-shaped molecules have distinct mobilities. Further analysis of the electrophoretic mobilities of DNA fragments of different shapes will be required to develop a quantitative basis for the interpretation of effects of the lengths of spacer and flanking sequences on electrophoretic mobility.

Phasing analysis of truncated Fos–Jun heterodimer binding to the his3-189 site demonstrated significant differences in complex mobilities. These differences were in part due to an intrinsic DNA bend at the his3-189 site. This site contains a run of 10 consecutive A·T base pairs that may contribute to this intrinsic bend. Further studies of DNA bending by Fos and Jun at this and other binding sites is necessary to determine the sequence dependence of DNA bending.

Ligase-catalyzed cyclization provides a powerful method for the analysis of the relative proximities of correctly aligned intramolecular and intermolecular DNA ends (26–28). However, many factors can influence the relative rates of cyclization and multimerization. The failure to detect the intrinsic bend at the his3-189 site (11) also suggests that this method may not be sensitive to small DNA bends. Truncated Jun homodimers can stimulate the cyclization of oligonucleotides in a manner that is dependent on the phasing of adjacent DNA bends. However, both homo- and heterodimers can stimulate the multimerization of restriction fragments regardless of the presence of adjacent bends. Previous studies using restriction fragment substrates found no effect of Fos and Jun on the relative rates of cyclization and multimerization (11). One possible interpretation of these results is that truncated Fos and Jun influence ligation through multiple mechanisms. On probes containing multiple DNA binding sites, the phase-dependent stimulation of cyclization is dominant. However, on long probes containing a single binding site, this effect is offset and, under some conditions, reversed, by a concentration-dependent stimulation of multimerization. One possible mechanism for this effect is the formation of tetramers or larger oligomers that may bind multiple DNA fragments and stimulate intermolecular ligation between the long flanking segments. This effect may be reduced or eliminated by the shorter flanking segments present on the probes used in oligonucleotide cyclization. Regardless of the mechanism for this effect, the multiple factors influencing the relative rates of inter- and intramolecular cyclization compromise the application of ligase-catalyzed cyclization for studies of DNA bending by Fos and Jun.

The results from phasing analysis appear to contradict not only the results from phase-sensitive detection and ligase-catalyzed cyclization (11), but also the results from x-ray crystallographic analysis (15) while they are supported by direct visualization of DNA bending by atomic force and electron microscopy (29). There are many differences among these experiments that may contribute to the disparate results. First, regions outside the minimal DNA binding and dimerization domains of Fos and Jun contribute to DNA bending (6, 10), and the small DNA bends caused by the bZIP domains alone may not be detectable by some methods. Second, the AP-1 sites used in the different studies have distinct flanking sequences, raising the possibility that the sequence of the binding site may influence DNA bending. Finally, the experimental conditions used in the various approaches are dramatically different. In particular, multivalent cations are often added to ligation reactions and crystallization buffers, but are not generally present during electrophoresis. These as well as other small molecules can affect intrinsic DNA bending (30–32) and may influence protein-induced DNA bending as well. By definition, crystallization requires constraint of the normal dynamics of the complex, which in many cases, including the Fos′–Jun′–AP-1 complex, involves end-to-end stacking of the binding site oligonucleotides. Such end-to-end stacking is likely to severely restrict the DNA conformations available to the complex. Further studies of the effects of these and other variables on DNA bending will be required for determination of the cause of the differences between DNA structures observed in different experimental paradigms.

I thank Tom Curran for unwavering support and encouragement, Nirmala Rajaram for construction of the his3-189 phasing analysis plasmids, Dmitry Kuprash and Sergei Nedospasov for plasmids A6.A2.42 and A6.A2.47, Donald Crothers and Ayesha Sitlani for sharing results before publication, and Mark Glover, Len Lutter, John Mueller, and David Paolella for constructive comments on the manuscript.

1. Gartenberg, M. R. & Crothers, D. M. (1988) *Nature (London)* **333,** 824–829.
2. Moitoso de Vargas, L., Kim, S. & Landy, A. (1989) *Science* **244,** 1457–1461.
3. Giese, K., Cox, J. & Grosschedl, R. (1992) *Cell* **69,** 185–195.
4. Falvo, J. V., Thanos, D. & Maniatis, T. (1996) *Cell* **83,** 1101–1111.
5. Gartenberg, M. R., Ampe, C., Steitz, T. A. & Crothers, D. M. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 6034–6038.
6. Kerppola, T. K. & Curran, T. (1991) *Science* **254,** 1210–1214.
7. Avitahl, N. & Calame, K. (1994) *J. Biol. Chem.* **269,** 23553–23562.
8. Kuprash, D. V., Rice, N. R. & Nedospasov, S. A. (1995) *Nucleic Acids Res.* **23,** 427–433.
9. Zinkel, S. S. & Crothers, D. M. (1987) *Nature (London)* **328,** 178–181.
10. Kerppola, T. K. & Curran, T. (1991) *Cell* **66,** 317–326.
11. Sitlani, A. & Crothers, D. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 3248–3252.
12. Fisher, D. E., Parent, L. A. & Sharp, P. A. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11779–11783.
13. Wechsler, D. S. & Dang, C. V. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 7635–7639.
14. Ferre-D'Amare, A., Prendergast, G. C., Ziff, E. B. & Burley, S. K. (1993) *Nature (London)* **363,** 38–45.
15. Glover, J. N. & Harrison, S. C. (1995) *Nature (London)* **373,** 257–261.
16. Kerppola, T. & Curran, T. (1995) *Nature (London)* **373,** 199–200.
17. Kerppola, T. K. & Curran, T. (1991) *Curr. Opin. Struct. Biol.* **1,** 71–79.
18. Thompson, J. F. & Landy, A. (1988) *Nucleic Acids Res.* **16,** 9687–9705.
19. Abate, C., Patel, L., Rauscher, F. J., III, & Curran, T. (1990) *Science* **249,** 1157–1161.
20. McCaffrey, P. G., Luo, C., Kerppola, T. K., Jain, J., Badalian, T. M., Ho, A. M., Burgeon, E., Lane, W. S., Lambert, J. N., Curran, T., Verdine, G. L., Rao, A. & Hogan, P. G. (1993) *Science* **262,** 750–754.
21. Kerppola, T. K. & Curran, T. (1994) *Oncogene* **9,** 3149–3158.
22. Shrader, T. E. & Crothers, D. M. (1990) *J. Mol. Biol.* **216,** 69–84.
23. Zinkel, S. S. & Crothers, D. M. (1991) *J. Mol. Biol.* **219,** 201–215.
24. Lumpkin, O. J., Dejardin, P. & Zimm, B. H. (1985) *Biopolymers* **24,** 1573–1593.
25. Levene, S. D. & Zimm, B. H. (1989) *Science* **245,** 396–399.
26. Ulanovsky, L., Bodner, M., Trifonov, E. N. & Choder, M. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 862–866.
27. Crothers, D. M., Drak, J., Kahn, J. D. & Levene, S. D. (1992) *Methods Enzymol.* **212,** 3–29.
28. Kahn, J. D. & Crothers, D. M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6343–6347.
29. Becker, J. C., Nikroo, A., Brabletz, T. & Reisfeld, R. A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 9727–9731.
30. Brukner, I., Susic, S., Dlakic, M., Savic, A. & Pongor, S. (1994) *J. Mol. Biol.* **236,** 26–32.
31. Dlakic, M. & Harrington, R. E. (1995) *J. Biol. Chem.* **270,** 29945–29952.
32. Sprous, D., Zacharias, W., Wood, Z. A. & Harvey, S. C. (1995) *Nucleic Acids Res.* **23,** 1816–1821.