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Commentary

Do basic region–leucine zipper proteins bend their DNA targets . . . does it matter?

Paul J. Hagerman

Departments of Biochemistry, Biophysics, and Genetics, B-121, University of Colorado Health Sciences Center, 4200 East Ninth Avenue, Denver, CO 80262

Protein-induced bending of DNA has been described for numerous members of the large family of "basic region–leucine zipper" (bZIP) transcriptional modulators (1–4). However, for this class of proteins, there exists some uncertainty as to how much (or even whether) the bound target DNA is bent. This issue has been most sharply focused for the bZIP protein heterodimer, Fos–Jun, with two recent articles in the *Proceedings* (5, 6) presenting seemingly disparate views as to whether its recognition element (AP-1) is (6)—or is not (5)—bent.

It has been known for some time that certain transcriptional regulators are capable of bending their DNA targets. For example, both solution (7) and crystallographic (8) studies of the *Escherichia coli* catabolite activator protein (CAP)–DNA complex suggest that CAP induces a ≈90° bend in its recognition sequence. A distortion of similar magnitude, albeit of very different local geometry, has been observed for a TATA box-binding polypeptide (9, 10). Distortions of this magnitude are highly improbable as thermal fluctuations; thus, one function of modulators such as CAP and TATA box-binding polypeptide may be to bring together otherwise distant DNA segments via protein-induced bends. In fact, tests of this general hypothesis have in some instances demonstrated that the specific function of protein-induced bends can be subserved either by heterologous protein-induced bends or by intrinsic (sequence-directed) elements of DNA curvature (11).

In 1991, Kerppola and Curran (12) reported that Fos–Jun heterodimers substantially distort the AP-1 site. Comparing the relative gel mobilities of DNA molecules in which the AP-1 site was placed at various positions (permuted) within the molecules (circular permutation assay; refs. 13–15), those authors reported "flexure" angles of 94° for full-length Fos–Jun, and 55° for a truncated form of the heterodimer (12). However, Kerppola and Curran (16) also performed a different form of gel experiment in which the AP-1 site was placed in varying torsional alignments with respect to a reference (sequence-directed) bend (phasing assay; ref. 17). This latter assay yielded 23° for the Fos–Jun-induced bend and 10–12° for the bend induced by the heterodimers. Furthermore, using the phasing assay, Kerppola and Curran (16) observed that the direction of the bend induced by Fos–Jun was nearly opposite to the direction created by the Jun–Jun homodimer, and they suggested that this directionality could contribute to the differential response of the glucocorticoid response element in the presence of Jun (activation) or Fos–Jun (repression).

However, using a combination of gel and solution methods, Sitlani and Crothers (5) reached quite a different conclusion regarding the Fos–Jun-AP-1 interaction, namely, that Fos–Jun does not bend its target (i.e., by less than 5°).

Thus, there actually appear to be two sources of disagreement: one involves a large disparity in the apparent angles derived from permutation and phasing methods; a second involves inconsistent results from phasing and cyclization assays. Since both gel and solution approaches continue to enjoy widespread use, and since the question of Fos–Jun-induced bending will necessarily turn on fine points of experimental method, it is worthwhile to consider each method. The details will turn out to be important, not only for Fos–Jun and other members of the bZIP family, but for the study of protein-induced bending of DNA in general.

The Circular Permutation Assay

Crothers and coworkers (13, 14) devised a means for locating intrinsic bends in DNA that involves a comparison of the gel mobilities of linear DNA molecules that differ from one another only by the circular permutation of their sequence—the molecule with the lowest mobility has the (net) bend at its center. The relationship between bend position and mobility was not unexpected, since reptation models (19–21) have held that the mobility (μ) of a molecule of length L is proportional to its mean-square end-to-end length. Thus,

$$\mu = \frac{\langle \dot{x}_{CM} \rangle}{E_x} = \frac{Q}{\xi} \frac{\langle h_x^2 \rangle}{L^2}, \qquad [1]$$

where h_x^2 is the component of the end-to-end vector in the direction (x) of the field (E_x), Q is the effective charge, and ξ is the frictional coefficient of the reptating molecule. Thompson and Landy (15) later quantified the relationship between mobility and end-to-end distance for a rigid, once-bend rod, obtaining the relationship,

$$\mu_\alpha / \mu_{linear} = \cos(\alpha/2), \qquad [2]$$

for the relative mobilities of bent and linear DNA molecules, where α is the bend angle. Thompson and Landy (15) stressed that the functional form of Eq. **2** was purely empirical—a way to relate relative mobilities of test molecules to a set of DNA standards possessing intrinsic curvature.* However, apart from its use as an interpolation function, there is little foundation for Eq. **2**; indeed, electrophoretic simulations (22) suggest that end-to-end distance is not the appropriate parameter for relating relative mobility to the bend angle. In fact, alternative models suggest that both bend angle and bend dispersion are more appropriate determinants of mobility (23); that is, mobility will decrease as the energy required to straighten the molecule increases.

Eq. **2**, or variants thereof, has been used widely for assigning angles to protein-induced bends via the permutation approach. For this extension, electrophoresis theory is almost completely silent. In particular, for many of the larger DNA binding proteins, the dominant influence on mobility is the physical size/shape of the protein moiety. It is not known how mobility should vary with protein position and size, even in the absence of a bend. This issue is particularly vexing for the bZIP proteins, where the angles determined by the circular permutation method may be too large by more than an order of

*Eq. **2** does not follow from Eq. **1**, since a linear relationship between mobilities and end-to-end separation is only true in the limit of a random-walk chain; one would expect a $\cos^2$-dependence in the rigid-rod limit. Moreover, the DNA molecules used for most permutation assays do not approximate rigid rods.

magnitude in some instances (3). This latter problem has prompted Kerppola and Curran (3) to declare that the circular permutation method "is not a reliable method for the determination of directed DNA bends." Their sentiment echoes that of others (24), who found a similar disparity for the GCN4 (bZIP)–DNA complexes. Since there is no way to know *a priori* to what extent a protein will influence mobility in ways other than through bending *per se* (e.g., direct size effects, extent of binding during the gel run,† etc.), the circular permutation assay should probably not be used for the purpose of quantifying protein-induced bends. Thus, discounting the values of 94° and 52° (full-length Fos–Jun and bZIP domains, respectively), one is left with an accounting of the difference between 23° (10° for truncated Fos–Jun) and <5°.

Phasing Analysis (Phase-Sensitive Detection)

A second gel method, phase-sensitive detection (17), is also used extensively to study protein-induced bending. In this method, a bend of known direction and magnitude (typically a set of A-tract elements) is placed near a "test" bend, with the number of base pairs separating the reference and test bends varied over one or more helical turns. The molecule displaying the lowest mobility presumably has the two bends in *cis*. One advantage of this approach is that the position of the protein with respect to the ends of the molecule is nearly constant, thus reducing the position dependence of mobility due to the protein itself. In this regard, Kerppola and Curran (3) have made the important observation that there is no correlation between the apparent bend angle from phasing analysis and the protein mass. For example, the activating transcription factor 2 (ATF2) homodimer (≈110 kDa), one of the largest bZIP dimers investigated thus far, leaves its DNA target essentially unbent.

Kerppola and Curran (3, 4, 16) have developed a variant of the phasing analysis by which they can extract angles from the phase-dependent variation in mobility, using a reference bend of known angle direction and magnitude. They have derived formulae for the mobility variation that incorporate a decay of the phasing amplitude with increasing bend separation. Although their model neglects intrinsic flexibility of the DNA (decreasing phase coherence with increasing separation length) and also reprises the cosine relationship between mobilities and bend angles, they do observe a striking similarity between two intrinsic bends of variable spacing and one intrinsic bend and one AP-1 site bound with truncated Fos–Jun heterodimer (6). Thus, it appears that the Fos–Jun heterodimer does, in fact, bend its target, but by exactly how much is difficult to determine with precision from the gel analysis.

But what about the observation of Sitlani and Crothers (5) that phase-sensitive detection yielded little or no variation? In a direct examination of this issue (6), Kerppola has provided an elegant demonstration that the results of the phasing analysis are sensitive to the separation between test and reference bends and to the length of the flanking DNA segments. In particular, Kerppola (6) demonstrates that there is no real disagreement for the phasing studies, since the shorter flanking sequences used by Sitlani and Crothers (5) would not have been able to detect the relatively small Fos–Jun-induced bends.

The DNA Cyclization Assay

In the ligase-catalyzed cyclization assay (27), one obtains an effective concentration of one end of a DNA molecule in the vicinity of the other end, where the two ends are appropriately aligned for ligation (Fig. 1). This concentration, termed J (Fig. 2) is a sensitive measure of both intrinsic helix flexibility (27, 31) and the presence of bends (28, 32). The cyclization method

†Proteins whose rates of association/dissociation are much faster than the time required for electrophoresis may nevertheless remain associated with the migrating DNA through "cage" effects (25, 26). In this situation, the fractional time that the DNA is bent may also be a significant factor in the position-dependence of mobility for the circular permutation approach. Thus, mutant proteins may appear to bend their targets to a lesser degree than the wild-type protein, when, in fact, the difference may be due to lower fractional occupancy during the gel run.

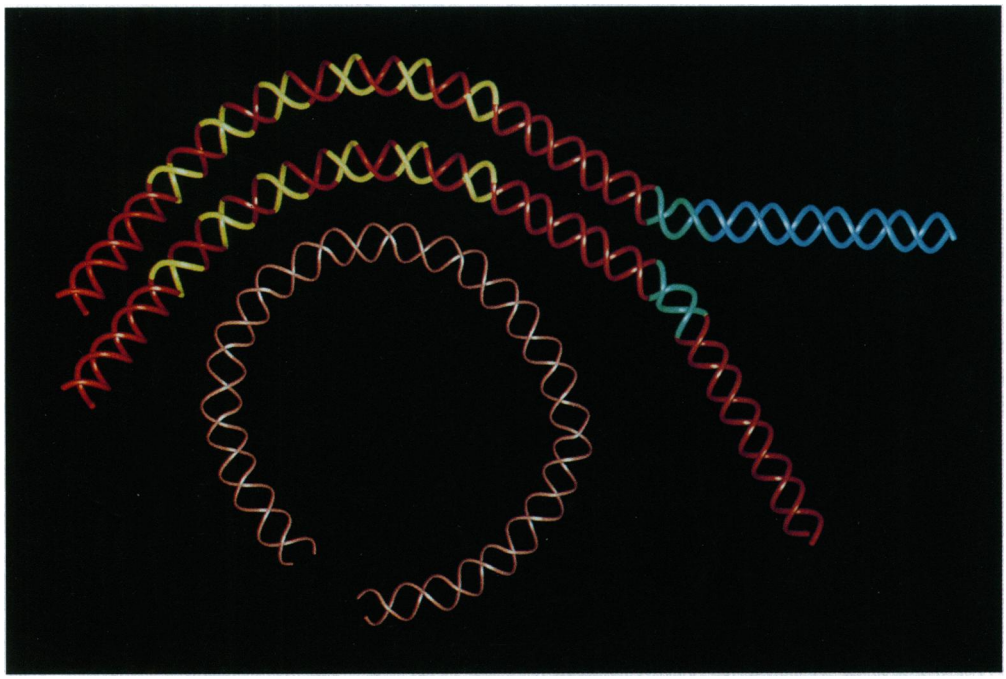

FIG. 1. Ribbon representations of the 158-bp DNA molecules used in the cyclization experiments of Sitlani and Crothers (5). The upper two helices depict alternative phasing arrangements between the intrinsic (A-tract) bends (yellow) and the AP-1 site (green). The cyclization assay effectively measures the propensity of a DNA molecule to form circular conformers (pink); this propensity is increased when the intrinsic and protein-induced bends are in *cis*.

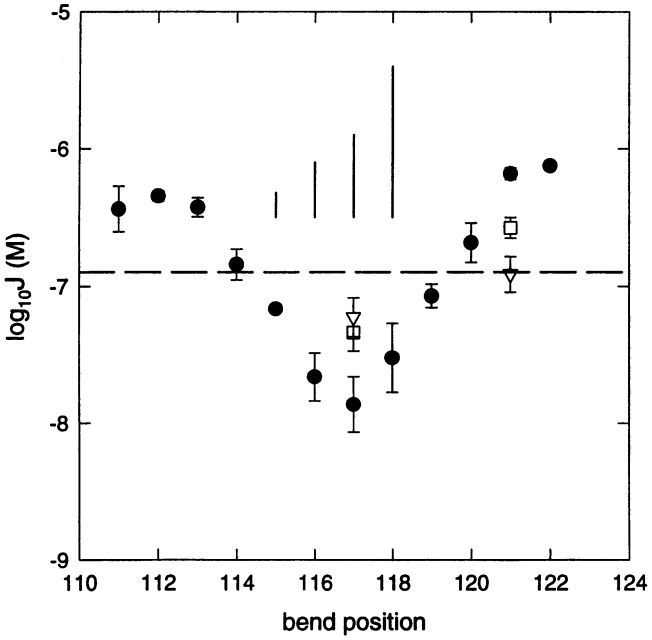

FIG. 2. Computed values of the cyclization propensities, J (molar), as a function of the base pair position of the center of the AP-1 site (5′-TGACTCA-3′), numbering from the 5′ end of the sequence, 17A9, in figure 1 of Sitlani and Crothers (5); the locations of the A-tracts are also specified by that sequence. The current figure is intended only to illustrate the variation in J expected for the 158 bp molecules for bends of 20° (●), 10° (□), 5° (▽), or 0° (- - -) at the AP-1 site; no effort has been made to fit the data of Sitlani and Crothers (5). Each point represents full J value computations (28) using ensembles of 3–7 × 10^{10} chains (standard errors as indicated) using the following helix parameters: A-tract bend, 18°; persistence length, 450 Å; torsional elastic constant, 2.5 × 10^{-19} erg-cm; helix repeat, 10.5 bp per turn. The vertical bars (left to right) represent the following variations of J: 1.5×, assumed measurement error; 2.5×, range of J values in the presence versus absence of Fos–Jun (5); 4.3×, variation in J due to intrinsic curvature in the "non-A-tract" region (5); 13×, variation in J due to intrinsic curvature for a related set of molecules (29). The variation in J expected for various phasings of the $T_9(A_9)$ tract located immediately 3′ to the AP-1 site approaches the computed variation (●) for the 20° bend (30).

has now been used for the purpose of quantifying both intrinsic and protein-induced bends (33–39). One major advantage of this assay as a solution-based approach is that it is free of the uncertainties associated with the gel-based approaches.‡

Kahn and Crothers (29) have introduced a novel variation of the basic cyclization approach by adding a set of internally phased, intrinsic bends into the DNA molecules being cyclized. In this instance, the precise value and nature of the intrinsic bend angles are less important than the fact that J is dramatically increased for all of the DNA molecules in the set. Thus, shorter molecules can be used, with the result that the effect of a small *additional* bend (in this case, the bound AP-1 site) will be magnified. For the 158-bp fragments used by Sitlani and Crothers (5) (Fig. 1), a 20° bend, phased through one helical turn with respect to the intrinsic (A-tract) bend should result in a *ca.* 50-fold variation in J, easily detectable if present (Fig. 2).

‡Both Sitlani and Crothers (5) and Kerppola (6) observed an influence of the bZIP proteins on the rates of both cyclization and bimolecular ligation reactions, which would tend to limit the accuracy of the J factors; however, these effects are less likely to affect the phase-dependence of the J values, whence bend-angles are derived. An additional confounding factor in the comparison of cyclization and gel results is the use of very different buffer conditions for the two types of experiments; this problem is widespread.

However, the high degree of sensitivity of the cyclization analysis renders it subject to other sources of curvature within the non-A-tract region. For example, the 158-bp sequences used by Sitlani and Crothers (figure 1 in ref. 5) possess several additional positions of curvature, including two additional AATT elements, each associated with bends of 5–10° (40), and a $T_9(A_9)$ element with a bend of 10–15° (30) in some constructs. These elements give rise to a 4-fold variation in the absence of Fos–Jun (10). Similar constructs for the earlier CAP studies (29) gave variations of up to 13-fold in the absence of CAP.

Finally, since the presence or absence of the $T_9(A_9)$ element ought to give rise to variations as large as 50-fold for appropriately phased constructs, the observed 2-fold variation +/− $T_9(A_9)$ suggests that other sources of intrinsic curvature are modulating the net curvature within the non-A-tract region. Therefore, the observed variation of up to 2.5-fold§ in J in the presence versus absence of Fos–Jun suggests that a bend of 5–15°, while not confirmed, cannot be excluded. In other words, both groups agree that the bends induced by Fos–Jun are small, but the exact angle remains in doubt. It is also clear that the cyclization assay, at present a preferred approach for quantifying bends in solution, will require an additional level of refinement—a full set of measurements over one helix repeat for fragments in which other sources of curvature have been minimized—to reliably extract angles in the 5–20° range.

. . . and Does it Matter?

The apparent disagreement over the degree to which the Fos–Jun heterodimer—and by extension, many other bZIP proteins—bend DNA has served to highlight one important, general feature of bZIP proteins, namely, that they appear to induce, at most, modest bending of their DNA targets. For the majority of the full-length and truncated dimers investigated thus far, the global bend angles (i.e., spanning the entire AP-1 or ATF/CREB sites) probably do not exceed ≈10°, with the remainder probably not exceeding ≈20–30° (3, 4, 41), although exceptions may yet exist. To place these numbers in perspective, thermal fluctuations within the unconstrained DNA helix (persistence length ≈450–500 Å) (42) lead to rms angles per base pair of ≈7° (or 15–20° for a region spanning the AP-1 or ATF/CREB targets). In other words, for many of the bZIP protein–DNA interactions, the free energy required to reach the reported bend angles does not exceed kT. In this regard, the absence of an observed bend in the Fos–Jun (bZIP domain)–DNA complex in the crystal (43) may simply indicate that the observed end-to-end stacking of the DNA oligomers overwhelms any small amount of intrinsic curvature that might have been introduced by the protein. These small angles would appear to militate against the notion that the biological specificity of members of the bZIP protein family arises from differences in protein-induced bending *per se*; that is, whether bZIP proteins bend their targets by 0° or 10° is probably not important.

However, if the bZIP proteins are capable of introducing the same, or similar, small distortions in the context of a highly constrained/organized initiation complex, their effects would be much more profound. For example, inspection of Fig. 2 reveals that for a 158-bp loop, conversion from a +10° bend to a −10° bend would result in an order of magnitude difference in J, which, in effect, represents the difference in the likelihood of bringing two segments (separated by 158 bp) into appropriate juxtaposition. In fact, this example probably represents a lower bound, since the intrinsic curvature model assumes that the A-tract region is curved, but unconstrained. Thus, it

§From table 1 of Sitlani and Crothers (5), 9A17(hcg) ± Fos–Jun versus 13A13 ± Fos–Jun: 1.6/0.64 = 2.5.

is perhaps more appropriate to think of bZIP modulators acting in the context of a constrained protein–DNA complex, as might occur in the presence of TATA box-binding protein, HMG proteins (44), or even nucleosomes. The introduction of an additional 5–10° in such a constrained system could lead to significant transcriptional modulation.

Finally, in two additional respects precise knowledge of bZIP-induced bends is important. Such knowledge helps us to understand the nature of the protein–DNA interaction at the structural/thermodynamic level, as in the mechanism by which bZIP proteins (e.g., GCN4) can accommodate two different targets with nearly equal facility (41, 45). Moreover, the attention given to the refinement of both the gel-based and solution-based methods for determining protein-induced bends in nucleic acids, an important positive aspect of the current disagreement, will lead to improvements in both methods and both are needed.

When you get to a fork in the road . . . take it.

Yogi Berra

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