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Homodimer of p50 (NFκB1) does not introduce a substantial directed bend into DNA according to three different experimental assays

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ABSTRACT

Transcription factors can distort the conformation of the DNA double helix upon binding to their target sites. Previously, studies utilizing circular permutation—electrophoretic mobility shift assay suggested that the homodimer of p50 (NFκB1), canonical NF-κB (p65-p50), as well as several non-canonical NF-κB/Rel complexes, may induce substantial DNA bending at the binding site. Here we have applied three additional experimental approaches, helical phasing analysis, minicircle binding and cyclization kinetics, and conclude that the homodimer of p50 introduces virtually no directed bend into the consensus κB sequences GGGACTTTCC or GGGAATTCCC.

INTRODUCTION

Many DNA-binding transcription factors can distort DNA structure at the site of binding, in particular by introducing DNA bending (1–12). Other proteins may interact with essentially straight DNA (13–17). In the case of homo- and heterodimeric complexes, the extent of DNA bending may depend on the subunit composition and on the spacing between the corresponding half sites (7,16,18,19).

DNA bending has been implicated in several models for transcriptional activation (20–22) and may be considered as one of several possible signals delivered to the basal transcriptional machinery through a variety of activation pathways.

NF-κB/Rel is a family of pleiotropic transcriptional regulators known to be involved in the control of constitutive and inducible expression of numerous eukaryotic genes (23,24). These proteins can form various homo- and heterodimers, which can recognize the κB and κB-like target sequences with varying affinities (23,25). The documented role for the DNA sequence context of the κB site in the recognition (26–28) and, in particular, the effects of the nucleotide(s) located outside of the known binding sequence on the affinity (26), prompted us to investigate DNA bending properties of various NF-κB/Rel complexes. Previously, circular permutation–electrophoretic mobility shift assays (CP/EMSA) have suggested the existence of substantial and complex-specific DNA bends (29,30).

In this study we reinvestigated the presumptive bending of DNA induced by NFκB1 (p50) homodimers by several alternative techniques, including helical phasing analysis, minicircle binding and cyclization kinetics. All these assays were performed on various p50 preparations and consistently showed that this complex, contrary to the previous conclusion, does not induce substantial DNA bending.

MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides were prepared on the Applied Biosystems Model 392 synthesizer and were purified by gel electrophoresis before use. The following oligonucleotides were used in construction of plasmids (only one strand is shown, without cloning sites; κB consensus sequences are underlined):

PD	CGTTG<u>GGGAATTCCC</u>CACTCC
Ig	CAGAG<u>GGGACTTTCC</u>GAGAGG
mTNF#4	GGGCAT<u>GGGAATTTCC</u>CACTC
pd42	GGAGGCGCAGCGTTG<u>GGGAATTCCC</u>CA
pd44	GGGCGCAGCGTTG<u>GGGAATTCCC</u>CACT
pd46	GCGCAGCGTTG<u>GGGAATTCCC</u>CACTCC
pd49	GAGCGTTG<u>GGGAATTCCC</u>CACTCCCAC
pd51	GCGTTG<u>GGGAATTCCC</u>CACTCCCACGA
pd52	GGTTG<u>GGGAATTCCC</u>CACTCCCACGAG
pd57	<u>GGGAATTCCC</u>CACTCCCACGAGTTTCA
ig42	GGCATCTCAACAGAG<u>GGGACTTTCC</u>GA
ig44	GATCTCAACAGAG<u>GGGACTTTCC</u>GAGA

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ig46	GCTCAACAGAGGGGACTTTCCGAGAGG
ig49	GAACAGAGGGGACTTTCCGAGAGGCCA
ig52	GAGAGGGGACTTTCCGAGAGGCCATCT
ig57	GGGACTTTCCGAGAGGCCATCTGGCAG
A242	GGCAGGGTTTTTTGCCGTTTTTTCCGG
A247	GGTTTTTTGCCGTTTTTTCCGGAGAGG

The following pairs of PCR primers were used:

225L	CTCACTATAGGGAGACCC
226L	TTAGGTGACACTATAGAATAC
A6PCR3	ATACACATACTGCAGAGGTGACACTAT
A6PCR5	ATACGACTCATATGAGGGAGACC

DNA constructs

The pGendH vector for CP/EMSA was a modified, pGEM4-based version of pBend2 (31) which contained a *Hin*dIII site instead of an *Xba*I site and a *Pst*I site instead of the original *Hin*dIII (32). Synthetic oligonucleotide duplexes containing the IgκB site (GGGACTTTCC), the palindromic (PD) κB (GGGAATTCCC), or the TNF κB site #4 (GGGAATTTCC), were cloned into the *Hin*dIII site of the pGendH vector, giving rise to the pGd.Ig, pGd.PD and pGd.4 constructs.

The series of A6.PD vectors for helical phasing/electrophoretic mobility shift assay (HP/EMSA) were prepared by simultaneous ligation of: (i) the 114 bp *Nco*I–*Avr*II fragment containing six phased A-tracts derived from the 11A17 construct described by Kahn and Crothers (33), (ii) a 33 bp synthetic duplex with *Nco*I and *Avr*II cohesive ends containing the PD NF-κB site with variable spacers (pdNN series, where NN denotes the distance in nucleotides between the center of κB site and the center of the first A-tract) and (iii) the *Nco*I-digested pGendH vector. In the A6.Ig series, a 50 bp *Bgl*II–*Avr*II fragment of the A6.PD.46 construct was replaced with a 50 bp synthetic duplex containing the IgκB site and spacers of variable length (igNN series of oligos). To prepare the control vectors A6.A2.42 and A6.A2.47 in which the κB sequences were substituted with the two phased A-tracts, double-stranded oligonucleotides A242 and A247 were used in a similar way.

Preparation of labeled DNA probes

Probes for CP/EMSA and HP/EMSA were prepared by labeled PCR, as previously described (32,34). PCR reactions were carried out in the presence of 20 μM of each dNTP and 0.3–0.5 μM [α-^{32}P]dCTP (3000 Ci/mmole), so that a specific activity of 5×10^6 to 10^7 c.p.m./pmole was typically obtained. For CP/EMSA, the entire multiple cloning site of pGd.Ig, pGd.PD or pGd.4 was amplified from vector-specific oligonucleotides 225L and 226L, and the PCR products were then digested with various restriction endonucleases to produce the set of 152 bp fragments (Fig. 2A), which were then purified by gel electrophoresis prior to further analysis. For HP/EMSA, the A6.Ig and A6.PD series of plasmids and the A6PCR3–A6PCR5 pair of primers were used to amplify a 275 bp fragment in which the six phased A-tracts and κB site were located roughly symmetrically relative to the middle of the fragment (Fig. 3A).

Minicircle binding and cyclization probes

To generate cyclization probes of 146 or 229 bp, labeled fragments for HP/EMSA were digested with *Nco*I or *Pst*I, respectively, and then purified by gel electrophoresis. Minicircles were prepared by ligation of the same probes in the standard T4 ligase buffer (New England Biolabs), followed by gel purification prior to binding assays.

p50 preparations

Transfections of human 293 cells were performed by the calcium phosphate precipitation technique. The expression constructs p50-CMV were provided by A. Israel (Institut Pasteur) and P. Baeuerle (Freiburg University), and preparations of purified human p50 produced in *E.coli* were kindly provided by P. Baeuerle, A. Israel, C. Larson and G. Verdine (Harvard University).

Extracts and binding reactions

Whole-cell extracts were made from transfected 293 cells. For binding assays, labeled probe ($1–5 \times 10^3$ c.p.m. in 1–2 μl of 3 mM Tris–HCl, pH 7.5, 0.2 mM EDTA) and 0.5–2 μl of the whole-cell extract (5–10 μg of total protein) were incubated at room temperature in 10 μl reactions for 10–30 min in the presence of 1 μg poly(dI–dC) (Boehringer Mannheim) and 0.2 μg sonicated double-stranded salmon sperm DNA in the following buffer: 10 mM HEPES, pH 7.8, 60 mM KCl, 0.02% Triton-X100, 1 mM EDTA and 1 mM EGTA, 6% glycerol (Sigma). For supershift analysis, antibodies were added to the mixture prior to addition of the probe and preincubated for 5 min.

In the case of purified p50, Triton-X100 was omitted from binding buffer, and 10 μg BSA was added per reaction to inhibit non-specific binding.

Antibodies

Monospecific antibodies to distinct members of NF-κB/Rel family of proteins have been described (35).

Electrophoretic mobility shift assays (EMSA) and quantitative analysis

For EMSA 5–8 μl of binding reaction were loaded on running 4–8% polyacrylamide gels (acrylamide:bisacrylamide, 30:1, in 0.5 × TBE) and separated by electrophoresis at 10 V/cm for 3–24 h (gels were typically polymerized overnight and were pre-run for 3–4 h). Dried gels were quantified on Ambis or Molecular Dynamics Phosphorimagers.

Ligation rate measurements

Considerations for ligation rate measurements were as described (36) except that very low DNA concentration (<1 nM) was used so that the bimolecular association was negligible, and only relative cyclization rates for a family of related DNA fragments could be measured. Reactions were carried out either in EMSA binding buffer or in T4 DNA ligase buffer. In both cases, the optimal concentrations of p50 and of the ligase were determined in pilot analytical experiments. To ensure reproducibility and accuracy, ligase was added to a binding reaction in 10 μl of appropriate buffer in a timed manner. The reaction was stopped by addition of an EDTA/tRNA mixture, followed by deproteinization with phenol–chloroform. An aliquot of the aqueous phase was then directly loaded on a 4% acrylamide gel to separate linear starting material from the circular ligation product. The relative

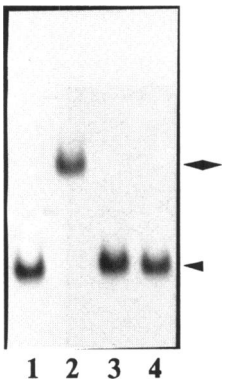

Figure 1. EMSA of p50 complexes in extracts from transfected 293 cells. The ^{32}P-labeled DNA probe was the 146 bp A6.PD.42/*Nco*I fragment used for generation of minicircles. Lane 1: no antibodies added; lane 2: anti-p50; lane 3: anti-p65 (RelA); lane 4: anti-p52 (NFκB2). Filled triangle: p50 complex; filled diamond: supershifted band. Free DNA is not shown.

cyclization efficiency either in the absence or in the presence of p50 was determined as a ratio of the amount of radioactivity in the circular form to that in the linear one. The latter was corrected for the fraction of 'unreactable' linear DNA determined in an experiment with a saturating amount of the ligase.

RESULTS

Identity of the p50 complex produced in transfected 293 cells

Whole-cell extracts from 293 cells transfected with the CMV-p50 expression construct were analyzed by EMSA. Single bands were observed with short oligonucleotide probes (not shown) or with longer probes designed for CP/EMSA, HP/EMSA and minicircle binding experiments (see below). The mobilities of these complexes were identical to those formed by purified p50 and the complexes could be supershifted only with anti-p50 monospecific antibodies but not with antibodies against other NF-κB/Rel family members (Fig. 1). Based on published data concerning the dimeric nature of the p50 complex (23,24,37,38), we concluded that the extracts of transfected 293 cells contained p50 homodimer and that the presence of other complexes containing endogenous NF-κB/Rel proteins could be neglected.

Homodimers of p50 from various sources induce equivalent effects in circular permutation/EMSA (CP/EMSA) assays

We first studied properties of the p50–DNA complexes in CP/EMSA assays in order to compare p50 from various sources and to verify previously published data.

This assay is based on the observation that a bend, either intrinsic or induced, in a DNA fragment affects its mobility in acrylamide gel (39–41). The maximum effect occurs when the bend is in the middle of the fragment, and the minimum effect is observed when it is at the end. To carry out this assay, a series of DNA fragments of identical sequence and length was generated, each of which had a κB binding site inserted at a different position (Fig. 2A). These ^{32}P-labeled fragments were individually incubated with a source of p50 homodimers and the mobility of the resulting complexes was determined (Fig. 2B). The results

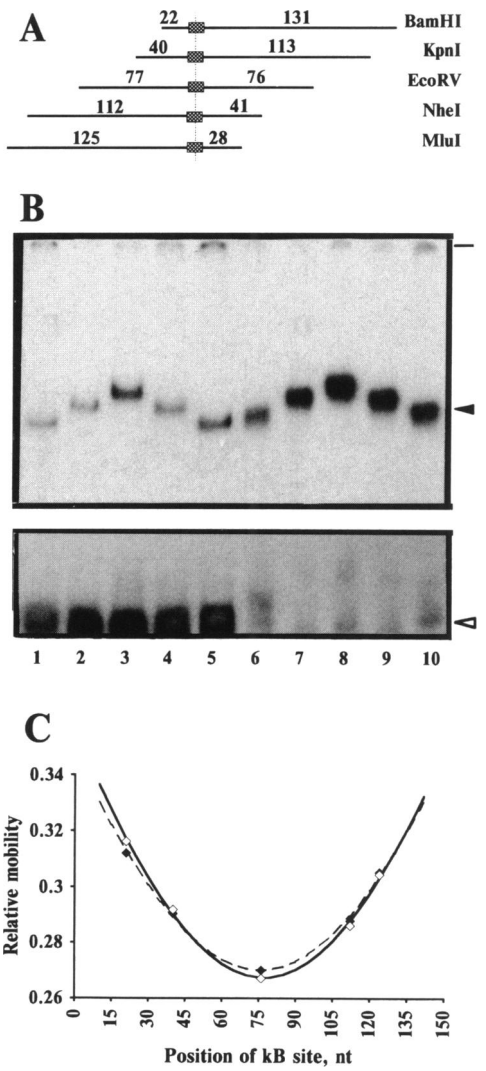

Figure 2. Circular permutation analysis (CP/EMSA) for p50 homodimers. (**A**) Probes used in CP/EMSA. Numbers show distances in nucleotides. The shaded boxes represent the κB site. (**B**) CP/EMSA patterns for different preparations of p50. Lanes 1–5: extracts from the 293 cells transfected with the p50 expression vector; lanes 6–10: purified p50. DNA probes used: lanes 1 and 6—pGd.4/*Bam*HI; lanes 2 and 7—pGd.4/*Kpn*I; lanes 3 and 8—pGd.4/*Eco*RV; lanes 4 and 9—pGd.4/*Nhe*I; lanes 5 and 10—pGd.4/*Mlu*I. The lower panel shows the migration of the free probes. Note that in lanes 6–10 almost all probe is bound. (**C**) Graphical representation of the results for the pGd.Ig (filled diamonds and dashed line) and pGd.PD (open diamonds and solid line) series.

indicated substantial position-dependent variation in the mobility, consistent with the earlier reports (29,30). Quantification of the mobility data (Fig. 2C) indicated 15% variation in the relative mobility (or 20%, if extrapolated to a hypothetical DNA fragment with κB site located at the very end), similar to the effects reported for p50 (NFκB1) homodimer and for canonical NF-κB (NF-κB1-RelA or p50-p65) (29). Side-by-side comparison (Fig. 2B) indicated that the complexes formed with the extracts and with purified p50 showed identical migration profiles, arguing against contribution of any additional components that may be present in the extracts of transfected 293 cells, such as HMG-I proteins (42) which can bend DNA and have been reported to be involved in multiprotein complexes on some κB sites (43). Similarly, the same position-dependent variation in the complex mobility was

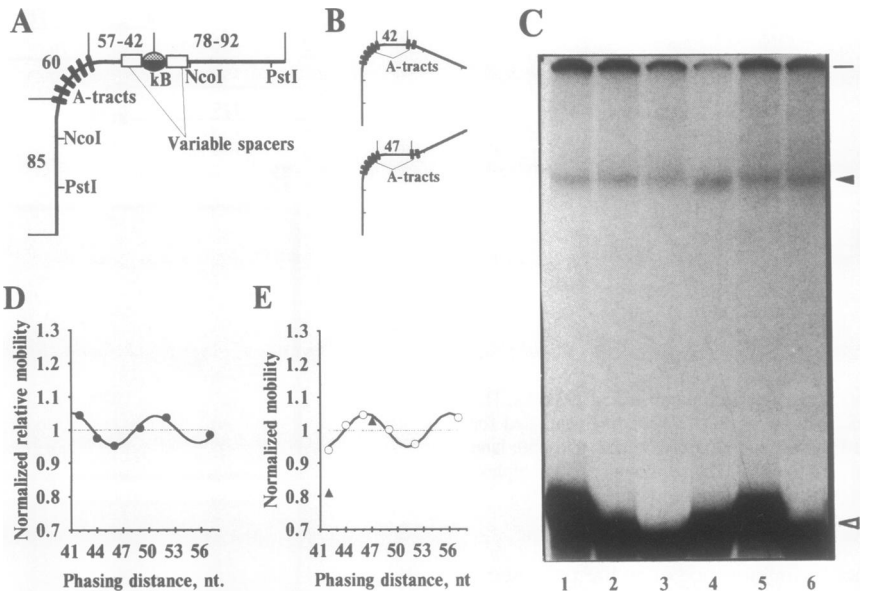

Figure 3. Helical phasing analysis (HP/EMSA) for p50 homodimers. (**A** and **B**) Constructs used for HP/EMSA. Numbers show distances in nucleotides. (**C**) An example of HP/EMSA pattern for p50 complexes from extracts of transfected 293 cells. DNA probes were of the A6.PD series, and the phasing distance was 42, 44, 46, 49, 52 and 57 bp for lanes 1, 2, 3, 4, 5 and 6, respectively. Open triangle, free DNA; solid triangle, p50–DNA complex. (**D**) Graphical representation of the HP/EMSA results for p50–DNA complex. Relative electrophoretic mobility of each individual complex was divided by the average relative mobility and plotted as a function of phasing distance. (**E**) Electrophoretic mobility of free HP/EMSA DNA probes, normalized against the average mobility of the A6.PD series of fragments. Open circles, A6.PD probes; solid triangles, A6.A2.42 and A6.A2.47 probes.

observed with three different κB sites: the Ig site, the PD site and the TNF site #4. The best fit of data to the empirical equation of Thompson and Landy (44) was obtained when the equivalent DNA bend was assumed to equal 80°, again in agreement with the published results (29).

Helical phasing analysis

The CP/EMSA assays may not distinguish between directed protein-induced bend and isotropic protein-induced DNA flexure (7). In addition, in some instances CP/EMSA has been reported to detect substantial mobility effects in the absence of any significant directed protein-induced bends (13) (as determined by other methods, including X-ray crystallography). Therefore, the complexes between DNA and p50 homodimers from various sources were subjected to helical phasing (HP/EMSA) analysis, which is designed to detect directed DNA bends (45) (see Fig. 3A).

In HP/EMSA, DNA with an intrinsic bend is employed. A binding site for the protein of interest is inserted into DNA, and one measures the ability of the bound protein to affect the overall bend of the DNA fragment and, therefore, its electrophoretic mobility in polyacrylamide gel (45). The protein-induced bend could be in the same direction as the intrinsic bend, in which case the two bends synergize, or it could be in a different direction, in which case they oppose.

Alternatively, there might be no protein-induced bend, in which case the overall bend is unaffected. In practice, when the direction of the test bend is unknown, a series of DNA fragments is generated, each of which contains a spacer of a different length between the intrinsic bend and the protein binding site. Synergy or opposition is expected to vary, depending on the length of that spacer.

In the constructs for HP/EMSA, we incorporated a 114 bp fragment from the 11A17 construct described by Kahn and Crothers (33). This fragment contains six phased A-tracts, which form a planar intrinsic bend (Fig. 3A); in several initial experiments the three A-tract constructs (7) were also used (data not shown). Interestingly, for the A6.PD series of constructs, the assay detected some variation in the mobility of free DNA, depending on the spacer length (Fig. 3C). Evidently the DNA itself contains some intrinsic bend apart from that of the A tracts. This effect was not seen when the same κB site and spacer elements were cloned into the pGendH vector (Fig. 2B).

In the presence of p50 homodimers, we observed only a very slight phase-related variation (5–10%) in the relative mobility of the protein–DNA complexes (Fig. 3C and D); this result was the same for different p50 preparations. If the results of the circular permutation analysis reflect p50-induced bending, a much larger effect than 5–10% is expected in HP/EMSA. If we assume: (i) that the mobility of a curved DNA fragment in 8% polyacrylamide gel is proportional to its end-to-end distance (44) and (ii) that a p50 homodimer is small compared to the size of the DNA fragment, then we expect the mobility variation in HP/EMSA to be 40% or more, taking into account the effect of chirality on DNA mobility in high percentage polyacrylamide gels (46). However, curve fitting of the data (Fig. 3D) suggests that very little directed bending occurs upon binding of the p50 homodimer to a κB site—15° or less, as compared to 70–80° estimated from CP/EMSA (29).

To test directly the sensitivity of our HP/EMSA assay, we prepared two control fragments, A6.A2.42 and A6.A2.47, in which κB sequences were substituted with the phased A-tracts

(Fig. 3B). The distances between the six- and the two-A-tract stretches were made equal to 42 and 47 nucleotides, or 4 and 4.5 helical turns, respectively, so that the two intrinsic bends were either in *cis* or in *trans* relative to each other (Fig. 3B). We expected the two A tract sequence which was intrinsically bent by about 36° (47) to cause much stronger modulation in the DNA mobility than that observed for p50–DNA complexes. Indeed, the data presented in Figure 3E indicate that the control bend of 36° was about twice as effective in producing a phasing effect as compared to the p50 homodimers, in agreement with the estimated upper limit of the p50-induced DNA bend of <15°.

Minicircle binding assay

An alternative technique to study protein-induced DNA bending is dependent on the utilization of pre-bent target sites that are placed in the context of minicircles (33). Proteins that induce DNA bending are expected to bind to minicircles containing optimally pre-bent sites with higher affinity as compared to linear probes. In this case, comparison of complexes formed on a set of minicircles with various orientations of the binding site should result in periodic modulation of binding affinity. We applied this technique to study modulation of p50 binding to various orientations of the κB site in order to shed additional light on the nature of protein-induced changes in DNA conformation.

We constructed two sets of κB-minicircles, of 146 and 229 bp in length, with seven different spacers separating the six A-tracts and the PD κB site, with corresponding spacers between the κB site and the *Nco*I site to maintain constant total length (see Fig. 3A). These seven variants (identified below by the length of the shorter spacer) corresponded to seven various configurations of the κB site, including its location on the outer, on the side, or on the inner surfaces of the minicircle.

The binding of p50 homodimer to a set of 146 bp minicircles is shown in Figure 4. The experiment reveals the absence of any periodic modulation in the affinity of binding. This was true for different incubation times (from 5 to 30 min, data not shown), making involvement of kinetics factors unlikely. Thus, there is no indication that p50 prefers any particular pre-bent DNA. This is consistent with the results of HP/EMSA and suggests that p50 induces no directed bend or unusual flexibility [see (34)] upon binding to the κB site.

On the other hand, minicircle binding assays did reveal phase-dependent variation in the *mobility* of the bound circles with various orientations of the binding site (Fig. 4), while control reactions with linear probes showed the same binding affinity but no modulation of mobility (data not shown). Similar experiments with a set of 229 bp minicircles resulted in a smaller variation in relative mobility of the complexes (data not shown). Although the nature of this mobility variation was not further studied, we favor the interpretation that this may be due to the position of the protein complex outside or inside the minicircle, with the latter having the higher mobility. If this interpretation is correct, the minicircle binding assay may prove to be a helpful technique in determining the orientation of the bound protein relative to the DNA double helix.

Cyclization kinetics assay

CP/EMSA, HP/EMSA and minicircle binding assays all depend on the migration of the complexes through a polyacrylamide gel matrix. An alternative technique to study protein induced DNA bending which is not dependent on PAGE is the comparative measurement of cyclization kinetics in solution in the absence and in the presence of bound protein. Moreover, the existing theory of ligation kinetics allows a quantitative interpretation of the data (33,36), if appropriate controls are used. A simplified version of this assay used here depends on the comparative measurement of the cyclization rates for a family of the variably spaced constructs (36).

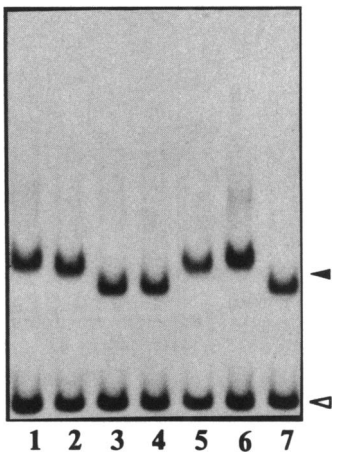

Figure 4. Minicircle binding assay for p50 homodimers. 146 bp circles of the A6.PD series bound to p50 derived from extracts of transfected 293 cells. Phasing distance: 42, 44, 46, 49, 51, 52 and 57 bp, lanes 1–7. Open triangle, free DNA; solid triangle, p50–DNA complex.

For the cyclization kinetics assay, we used the same 146 and 229 bp DNA fragments (derived from the A6.PD series of constructs) that were employed in the minicircle binding experiments; each contained six A-tracts, which cause an intrinsic bend. These fragments were ligated in the absence or in the presence of p50 homodimers (purified, or from the extracts of transfected 293 cells). In the presence of a protein-induced DNA bend, those constructs in which the two bends are in a *cis* orientation are expected to circularize faster than the corresponding controls without protein, while the *trans* molecules will circularize slower. In the well-studied case of bacterial CAP protein, the observed difference between *cis* and *trans* molecules was as large as two orders of magnitude (33). Since the design of our constructs is very similar to that of Kahn and Crothers (33), we find it reasonable to use the above value as an estimate of the expected effect. Conditions were optimized to ensure the linear dependence of the rate of appearance of circularized products on ligase concentration, and partition of the DNA between circular and linear forms in the range of 10–50%, suitable for quantification. Almost no formation of linear multimers was observed under our ligation conditions.

The results of the experiments are shown in Figure 5 and indicate that neither the 229 bp (*Pst*I digested) nor the 146 bp (*Nco*I digested) sets of probes exhibited any substantial effect in the presence of the bound p50 homodimers. It was true both for purified p50 and for p50-containing extracts from transfected cells. In fact, the most significant phase-dependent variation in the cyclization kinetics within the set was observed for the 146 bp set without protein, consistent with the notion that PD κB site possesses a small intrinsic bend (32). The substantial overall inhibition of ligation by p50 observed for the 146 bp set, was most

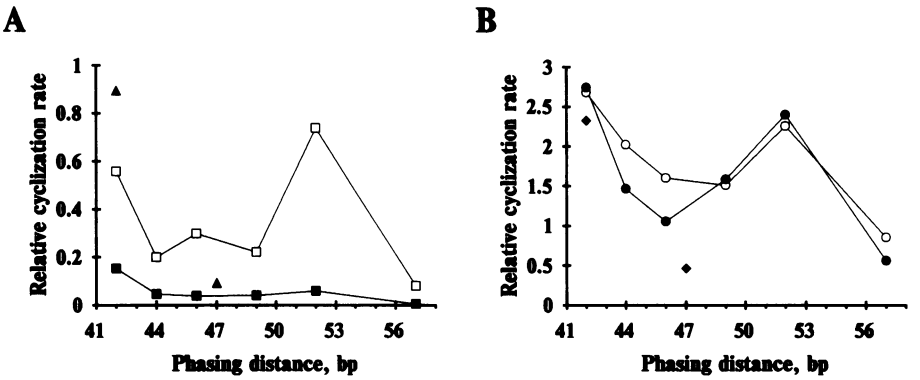

Figure 5. Cyclization kinetics assays in the absence and in the presence of p50 homodimers. Phasing probes A6.PD.42, 44, 46, 49, 52 and 57 were used for analysis. (**A**) Relative cyclization rates of the 146 bp (*Nco*I digested) DNA probes: open squares, A6.PD series; solid squares, A6.PD series in the presence of p50; solid triangles, control fragments A6.A2.42 and A6.A2.47. (**B**) Relative cyclization rates of the 229 bp (*Pst*I digested) DNA probes: open circles, A6.PD series; solid circles, A6.PD series in the presence of p50; solid diamonds, A6.A2.42 and A6.A2.47 fragments. Concentration of the ligase in (B) was 15 times lower than that in (A).

probably due to p50 binding at the very end of the test fragment (Fig. 3A), and this does not affect interpretation of the results.

The results shown in Figure 5 indicate that the magnitude of p50-induced bend, if any, at the PD κB site, is smaller than that of the intrinsic DNA bend at this site. Since this intrinsic bend was undetectable in our CP/EMSA experiments (data not shown), its magnitude is ≤15°, which is the limit of sensitivity of CP/EMSA in our hands. Therefore, these results provide independent evidence that the p50 homodimer does not induce substantial directed DNA bend upon binding to the κB site and they impose an upper limit of 15° on the magnitude of any p50-induced bend.

This conclusion is not limited to the palindromic κB site used in our cyclization experiments. Cyclization assays with p50 on the MHC kB site GGGGATTCCCC also show no evidence of a directed bend (C. Larson and G. Verdine, personal communication).

Finally, the sensitivity of our cyclization assay was tested directly using control constructs A6.A2.42 and A6.A2.47 (Fig. 3B). The results of this assay indicate that both in the case of 146 bp fragments (Fig. 5A) and 229 bp fragments (Fig. 5B) the two A-tract sequence bent by 36°, produced phase-dependent variation in cyclization rate, and this effect was larger than that of the PD κB site, either intrinsically bent or occupied by p50 homodimer. Thus, the conclusion drawn from the cyclization experiments in the presence of p50, was further confirmed.

DISCUSSION

This study was aimed at understanding possible conformational changes in DNA upon binding of p50 homodimer to its cognate κB site. Our results provide three lines of evidence against the existence of any substantial directed DNA bend, induced by the binding of p50 homodimer. Two of the assays were based on electrophoresis of the complexes in polyacrylamide gels, while the third assay was done entirely in solution.

In previous studies (29,30) CP/EMSA data were interpreted as indicating a high extent of DNA bending by various NF-κB complexes. In this study the CP/EMSA effects for p50 homodimers were reproduced with extracts from transfected 293 cells and with purified p50, but our different conclusion, on the absence of any substantial directed protein-induced DNA bend, is based on the use of three additional alternative techniques: helical phasing analysis, binding of p50 to phased minicircles, and cyclization kinetics.

To account for the substantial effects in CP/EMSA for Jun- and Fos-containing complexes with DNA, the existence of a flexible hinge has been proposed (7,48). Although this 'flexure' model is not ruled out, the results of the recently published study have shown that a model hinge in DNA produced by 3 bp mismatch could give rise to only relatively small effects in CP/EMSA and HP/EMSA assays, although the hinge can be easily detected in cyclization kinetics experiments (34). Lack of the overall increase in cyclization efficiency in the presence of p50 in our cyclization kinetics measurements (Fig. 5), also suggests that p50 does not induce substantial DNA flexure. Therefore, we find it unlikely that increased DNA flexure can reconcile the conflicting results obtained for p50 homodimers in CP/EMSA assay, on one hand, and in three other assays, on the other hand.

Mobility variations of p50–DNA complexes in CP/EMSA that, according to the results of the alternative techniques should not be interpreted as due to DNA bending, may result from the peculiar shape of the protein complex sitting on DNA. Indeed, in the case of CAP protein, for which all methods gave compatible results, the size of the protein component is about half that of the p50 homodimer, while the interaction region spans about 30 bp (10,11). p50 homodimer apparently interacts with only 10–11 bp, suggesting that the protein component of the complex may be extended away from the DNA path. Given these considerations, we would expect the results of CP/EMSA to be more reliable if larger DNA fragments are used.

The exact DNA conformation in all distinct NF-κB–DNA complexes will be revealed by direct methods, such as X-ray crystallography and 3-D NMR. In the absence of these data, indirect methods, such as HP/EMSA, minicircle binding and cyclization kinetics, now provide strong evidence that, at least in the case of p50 homodimers, DNA is not bent substantially. These results, however, do not exclude a possibility of other distortions in the DNA structure, such as DNA unwinding.

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