The NF-κB transcription factor induces DNA bending which is modulated by its 65-kD subunit

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ABSTRACT

This study describes a novel functional property of the eukaryotic transcription factor NF-κB in altering the DNA structure. We observed that the binding of purified NF-κB to DNA was facilitated by spermine and cations which are known to promote DNA bending, and by a nick in one position at the 3'-end of the binding site. Furthermore, the position of the NF-κB binding sequence 5'-GGGACTTTCC-3' (κB motif) within circularly permuted DNA fragments had a profound influence on the mobility of NF-κB-DNA complexes in a gel retardation assay while the mobility of unbound DNA fragments did not depend on the position of the κB motif. The mobility effect was slightly reduced at increased temperature. These observations suggested that binding of NF-κB to DNA induces bending. The estimated bending angle induced by the 50-kD DNA binding subunit of NF-κB (p50) was smaller than that induced by p50 which was associated with the 65-kD non-DNA binding subunit (p65). Moreover, the presence of p65 appeared to result in a shift of the bending center from the middle towards the 3'-end of the κB motif. This shows a role for the p65 subunit of NF-κB in modulating the extent and altering the position of protein-induced DNA bending.

INTRODUCTION

The NF-κB transcription factor can induce the expression of genes in different cell types by binding to decameric sequence motifs in promoter and enhancer elements (for reviews see 1–3). Most of its target genes encode proteins that are rapidly induced as part of immune and acute phase responses and during inflammatory processes. These proteins include immunoreceptors, acute phase proteins, cytokines and cytokine receptors. NF-κB can very rapidly activate genes because the protein is already present in unstimulated cells and requires for its activation only the release from its inhibitory subunit IκB and translocation from the cytoplasm into the nucleus (4). Viruses, T cell mitogens, bacterial lipopolysaccharide, cytokines and various other agents can activate the transcription factor (for reviews see 2,3). Phosphorylation of IκB by protein kinases appears to be an important trigger for the activation of NF-κB (5,6).

NF-κB exists as various multiprotein complexes. The simplest DNA binding form of NF-κB is composed of a dimerized 50 kd protein (p50) (7). It can be obtained from a heterotetrameric complex of NF-κB, which contains in addition to p50 two 65 kd non-binding subunits (p65), by electrophoretic separation of p50 under denaturing conditions followed by its renaturation. At present, it is unclear whether the p50 dimer has a physiological relevance or is an artifact. The prominent DNA-binding form of NF-κB in nuclei of activated cells is the heterotetramer. The non-DNA binding cytoplasmic NF-κB from unstimulated cells is a heterotrimeric complex composed of p50, p65 and IκB (8). Presumably, IκB prevents in this complex that two p50-p65 heterodimers can assemble to a heterotetramer. The p65 subunit serves as a receptor for the inhibitory subunit IκB (9) and is therefore required by NF-κB to become inactivated again by IκB. This can occur even when NF-κB is bound with high affinity to DNA (8).

An additional role of p65 in the nuclear form of NF-κB is to modulate the DNA-binding specificity of p50 (9). While, in the absence of p65, the p50 dimer can bind with high affinity to completely palindromic binding sequences, the heterotetramer binds with a 10- to 20-fold lower affinity to these sites but shows a two-fold increased affinity for the less symmetric κB motif 5'-GGGACTTTCC-3'. It is not yet understood how the multiprotein complex of NF-κB can initiate the synthesis of mRNA as a consequence of its binding to enhancer and promoter elements, and whether p65 is required for transcriptional activation.

In this study we have investigated whether binding of NF-κB alters the structure of DNA. A distortion of DNA structure that in prokaryotic systems is apparently involved in the control of transcription is DNA bending (10,11). Bending occurs either as an intrinsic property of DNA or is induced after sequence-specific binding of proteins (for reviews see 12–14). Here we report that DNA is strongly bent upon binding of NF-κB and that the non-DNA binding 65 KD subunit of NF-κB modulates the extent and alters the position of DNA bending.

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RESULTS

Polyamines and metallic cations facilitate binding of NF-κB to DNA

Using electrophoretic mobility shift assays (EMSAs), we have tested the influence of polyamines and various metallic cations on binding of NF-κB to an oligonucleotide encompassing the κB binding motif 5'-GGGACTTTCC-3' (Fig. 1A). The NF-κB was DNA affinity-purified from cytosol of human placenta after its activation by a low pH-treatment (8). It was composed of both 50 and 65 kD protein subunits (referred to as heterotetrameric NF-κB) and was indistinguishable from active nuclear NF-κB (15). In the EMSAs shown in Figure 1 (A and B, lane 1) a concentration of NF-κB was used which gave only a barely detectable amount of protein-DNA complex. A dramatic increase in the amount of the complex was obtained if spermine was included in the binding reactions (Fig. 1A, lanes 2–5). Optimal complex formation was seen with 0.5 mM spermine. Spermidine had a similar although weaker effect (data not shown). Spermine has been reported to promote and stabilize structural alterations of DNA such as bending (16). It is therefore possible that the increased binding of NF-κB to DNA in the presence of spermine resulted from a structural alteration of the target DNA sequence by the polyamine. Alternatively or in addition, polyamines could exert their effect by stabilizing the protein subunit structure of NF-κB.

We also tested various metal cations which have been reported to promote DNA bending (16,17) for their effect on the DNA binding of NF-κB (Fig. 1B). While 1 to 10 mM Mg2+ barely increased DNA-binding of the transcription factor (Fig. 1B, lanes 2 to 4), Ca2+, Ba2+ and Co3+(NH3)6 cations induced a significant dose-dependent increase in the amount of the NF-κB-DNA complex (lanes 5 to 13). The maximal effects were seen with 5 mM Ca2+, 1 mM Ba2+ and 25 μM Co3+(NH3)6. These concentrations were similar to those at which the metal cations were previously shown to exhibit optimal alteration of DNA structure. The enhancing effect by both polyamines and various metallic cations suggests that the interaction of NF-κB with DNA is facilitated by modification of the cognate DNA structure rather than by a direct interaction of the agents with the protein.

Selective binding of NF-κB to nicked DNA

Support for the idea that bending or kinking of DNA facilitates binding of NF-κB came from a hydroxyl radical missing contact analysis (19). This method allows to investigate the effect of specific nicks in the DNA backbone on the binding of proteins. For that purpose, the DNA was OH-radical treated prior to its use in EMSAs. It is not yet clear whether the OH-radical treatment solely nicks DNA or, in addition, removes a nucleoside. The 32P-end-labeled DNA fragment used in an EMSA (Fig. 2A, lane 1) showed a regular accessibility for hydroxyl radicals (Fig. 2A, lane 5; 2B, panel 1) and no strikingly unusual DNase I digestion pattern in the κB motif suggesting that the NF-κB binding site had no unusual structure prior to the binding of protein (19,20)(Fig. 2A, lane 2). The conditions for the OH-radical treatment were chosen such that on an average only a single nick per DNA molecule was produced. Among the OH-radical treated DNA-fragments that were bound by NF-κB, the amounts of those species were found decreased which were damaged by a nick within the decameric binding motif 5'-GGGACTTTCC-3' (Fig. 2A, lane 6; 2B, panel 2). There was no single position within this binding motif where a nick could completely prevent the interaction of NF-κB with DNA (Fig. 2B, panel 2). Missing contacts in the 3'-half site of the decameric motif interfered with binding of NF-κB more strongly than those in the 5'-half site, as is evident from the scan shown in Fig. 2B (panel 2).

A DNA fragment that was nicked in the next position downstream of the 3'-end of the binding motif was found highly enriched in the NF-κB-DNA complex and was present in a reduced amount in the fraction containing unbound DNA (Fig. 2A, compare lanes 6 and 7; 2B, compare panels 2 and 3). It appears that the binding of NF-κB to its cognate DNA was greatly facilitated if there was a nick at the 3'-end of the motif. The result with NF-κB is reminiscent to one obtained with the bacteriophage 434 repressor which recognizes an operator sequence with a single nick in its center with a higher affinity than the intact site (21).

NF-κB induces an asymmetric bending of the κB motif

The above observations prompted us to test directly whether NF-κB is capable of inducing a DNA bend or kink, or of increasing locally the flexibility of DNA. Circular permutation mobility shift assays (CP-EMSAs) were used in many instances to demonstrate that certain DNA sequences, or the binding of proteins to DNA, cause an alteration of DNA structure (22,23). An oligonucleotide encompassing the NF-κB binding site 5'-GGGACTTTCC-3' and flanking sequences from the mouse κ light chain enhancer was inserted into a vector allowing circular permutation of the site (24). Eight 146 bp long restriction fragments (A-H) were isolated from the vector pUC-κB-Bend which contained a single NF-κB binding motif in various positions (Fig. 3A). After 32P-endlabeling, the DNA fragments were used in EMSAs to form complexes with the heterotetrameric form of NF-κB. A strong
it became evident that the center of bending did not match with the position of a G residue the absence of which facilitated NF-xB binding.

effect of the position of the binding site on the mobility of the protein-DNA complex was evident (Fig. 3B). Fragments with the binding site in the middle formed complexes with NF-xB which migrated much slower than complexes containing fragments where the xB site was located closer to either end. The mobilities of fragments that bind NF-xB in the middle (E) or at the end (A) showed an almost two-fold difference indicating a strong alteration of DNA structure. When the DNA fragments were not bound to the protein they all showed a very similar mobility in the 8% polyacrylamide gel used (Fig. 2B). This result suggests that the permuted DNA fragments did not differ in their presumably linear structure and that the xB motif displayed no anomalous DNA structure on its own.

The specificity of NF-xB binding was tested in a competition analysis (data not shown). A 50-fold molar excess of an unlabeled double-stranded 30 bp oligonucleotide containing an homologous xB motif abolished the radioactive complex formation while the same amount of a 30 bp oligonucleotide with the mutated xB motif 5'-GGGAATCTAA-3' had no significant effect. A radioactively labeled 120 bp XhoI fragment from the pUC-Bend vector with no inserted xB oligonucleotide was not detectably bound by NF-xB.

A quantitation of the results from the CP-EMSA is shown in Figure 3C. The relative mobilities of NF-xB-DNA complexes of various mobilities, the open arrowhead the position of unbound DNA fragments. The data suggest that the heterotetrameric form of NF-xB containing the non-DNA-binding 65-kD subunit alters the DNA asymmetrically which can be caused by protein-induced melting or untwisting of DNA (26), we investigated the temperature dependence of the effects seen in CP-EMSA. The mobility difference seen with fragments A and E at 4°C (Fig. 3) was reduced by only 6% if the gel was run at 37°C (Tab. 1) demonstrating that NF-xB alters the DNA structure also at a physiological temperature. The weak sensitivity towards increasing the temperature is consistent with binding of DNA. If NF-xB would partially melt the DNA, an increased temperature should augment rather than decrease the difference in mobility between complexes formed with circularly permuted DNA fragments. Alterations of the run length and

![Figure 2](https://academic.oup.com/nar/article-abstract/18/22/6497/1054194/6497)

![Figure 3A](https://academic.oup.com/nar/article-abstract/18/22/6497/1054194/6497)

![Figure 3B](https://academic.oup.com/nar/article-abstract/18/22/6497/1054194/6497)

![Figure 3C](https://academic.oup.com/nar/article-abstract/18/22/6497/1054194/6497)
the electric field had no or only a minor influence (Tab. 1). The only parameter that substantially reduced the ratio of mobilities was a lower polyacrylamide concentration of the gel. Presumably, the larger pore size decreased the resolution of the gel for differences in the shape of the various protein-DNA complexes.

Distamycin, a drug binding to the minor groove (27), was incubated with the DNA prior to addition of heterotetrameric NF-κB. It did not interfere at concentrations between 2.5 μM and 2.5 μM with the effect of spermine or the formation of a NF-κB-DNA complex (data not shown). The drug also did not alter the mobilities of complexes formed by NF-κB with the circularly permuted DNA fragments. This indicates that minor groove contacts of NF-κB are irrelevant for high affinity binding and DNA bending.

**p65 can modulate the extent and position of DNA bending**

The experiments described so far were performed with the heterotetrameric form of NF-κB which contains in addition to two DNA-binding p50 molecules also two non-DNA-binding p65 molecules. We have in the following investigated the influence of the p65 subunit on the various effects of NF-κB in CP-EMSAs by using a form of NF-κB which is devoid of p65 (7). In the absence of p65, the p50 DNA-binding subunit can form a dimer in solution and is bound to DNA as a dimer. As is characteristic for many dimerized DNA-binding proteins, p50 can bind with high affinity to completely palindromic motifs (9).

We have tested in CP-EMSAs whether the asymmetric alteration of the xB motif found for the heterotetrameric form of NF-κB depends on the presence of p65. p50 dimer was obtained by renaturing p50 which was separated from p65 on an SDS-polyacrylamide gel (7, 9). Binding of the p50 dimer to the circularly permuted DNA fragments resulted in significantly smaller mobility differences than seen after binding of the heterotetramer. The mobility difference obtained with fragments that bind the p50 dimer in the middle or at the end was at most 1.25-fold (Fig. 4; Tab.1). As opposed to almost two-fold in the presence of p65 (Fig. 3). The binding angles (α) induced by the heterotetramer and p50 dimer were estimated by the empirical equation μM/μE = cosα/2 (28) from the ratio of the relative mobilities of complexes (μ) containing DNA fragments with the binding site in the middle (M) or at the end (E) (Tab. 1).

According to this approximation, the heterotetramer induced a bending angle in the order of 110° and the p50 dimer an angle of only 75° (Tab. 1). This shows that p65 increased the bending angle induced by p50.

Also the bending center obtained with the p50 dimer was distinct from that seen with the heterotetramer. It was located precisely between fragments D and E and thus overlapped with a position corresponding to the center of the xB motif (Fig. 4B). Apparently, the p50 dimer makes a more even contact with the binding motif than the heterotetramer, resulting in a more symmetric bending of the xB DNA motif. From these results we conclude that the non-DNA binding 65-kD subunit of NF-κB can also alter the site of bending.

**DISCUSSION**

This study provides evidence that binding of the NF-κB transcription factor alters the structure of DNA. The alteration is most likely a bend or kink of the otherwise linear target DNA which is located within or close by the decameric binding site for NF-κB depending on the association of the p50 dimer with the 65-kD subunit. DNA bending was evident from the effect that the position of the protein binding site within DNA fragments of identical length influenced the mobility of the NF-κB-DNA complexes. The relative temperature-independence of the mobility shifts indicated that the change in DNA structure induced by the transcription factor was a bending of DNA rather than a local increase in its flexibility. This is supported by the observation that the binding of NF-κB to DNA is facilitated in the presence of various agents known to promote and stabilize DNA bending.

The structural alteration induced by the p50 dimer could be a bend which stretches evenly over the entire decameric binding sequence or a kink which is localized in the center of the motif. It is also possible that two kinks induced by each of the two p50 molecules add up their angles depending on their distance to each other. In case of the heterotetramer, an additional kink at the 3'-end of the binding sequence could be induced which adds up its angle to that of a weaker central kink or to that of a kink located in the 5'-half of the motif. Alternatively, a bend or kink is shifted from the center towards the 3'-end of the motif and its angle is increased by the action of p65 onto p50.

The experiments shown in Figures 1 and 2 suggest that binding of NF-κB to linear DNA is energetically less favorable than binding to nicked or bent DNA, presumably because the
electrostatic repulsion of phosphate groups in the DNA backbone poses an energy barrier upon bending. When negative charges are neutralized by bound spermine or metallic cations, or the flexibility of the DNA is increased by a nick in the backbone, the energy requirement for bending is lowered and, as a consequence, binding of NF-xB to DNA is enhanced. By analogy with the DNA bend induced by the CAP protein (29), we assume that the NF-xB-induced DNA-bending requires and the bend subsequently stores free energy. We recently demonstrated that the inhibitory subunit of NF-xB, IxB, can effectively increase the dissociation rate of a high-affinity NF-xB-DNA complex (8). This involved the dissociation of protein-protein interactions in the DNA-bound heterotetramer by IxB. The velocity of such a reaction might be greatly enhanced by releasing the energy that is stored in the protein-induced bend.

The DNA-binding motifs of two other transcription factors that induce bending, CBF/SRF (30) and the papilloma virus type 1 protein E2 (31), show a striking similarity to that of NF-xB. CBF/SRF binds to the consensus sequence CC(A+T-rich)GG and the E2 protein to ACCN6GGT. The consensus of xB sites can be written as GG(A+T-rich)CC or GGNC6CC and thus differs from the other two sites only in the symmetry but not in the spacing of G and C clusters. The (p50 dimer can bind with high affinity to the xB motif 5'-GGAAATTCC-3' (9).) The consensus motif CNCNG(A+T-rich)CNCG for the bend-inducing heat shock transcription factor (32) seems to be more distantly related but again an A-T-rich central sequence of six nucleotides is flanked by conserved C and G residues in a palindromic arrangement suggesting that bend-inducing DNA-binding proteins have related cognate motifs.

The significance of DNA-bending for stimulation of transcription is unclear. In eukaryotic genes, bent DNA segments might act as cis-acting elements, as it has been demonstrated in prokaryotes (10,11). For instance, in the gal promoter of E. coli an intrinsically bent DNA fragment can functionally substitute for the binding site of the DNA-bending CAP transcription factor. Swap experiments in which NF-xB binding motifs are replaced by intrinsically bent DNA fragments and experiments investigating the correlations between the bending angle induced in a xB motif, the affinity of a motif and its cis-acting potential would allow to test the effect of DNA bends on transcriptional initiation in eukaryotes.

Most of the currently identified NF-xB binding sites are of reduced symmetry and consist of pentameric half sites which are distinct in their degree of sequence conservation (15). The more conserved half site A has the consensus 5'-GGGPaPuN-3' while the second half site B tolerates a much broader variation of sequence. In the xB motif 5'-GGGACTTTCC-3', which is most frequently found in enhancer elements, half site A appears to be recognized with higher affinity by the protein than half site B, as suggested by competition experiments using isolated xB half sites within identical flanking sequences. The relationship between the symmetry of NF-xB binding motifs and the affinity of binding, and the role of the p65 subunit for the sequence recognition by p50 was investigated in a subsequent study (8). It was shown that, if p50 is associated with the p65 subunit, the physiological xB motif 5'-GGGACTTTCC-3' is bound with a two-fold higher affinity whereas completely palindromic motifs are recognized with a more than ten-fold lower affinity by p50. The data suggested that the interaction of p50 with DNA is altered by the non-DNA binding 65-kD subunit of NF-xB. The effect of p65 on the p50-induced bending observed in the present study parallels the effect of p65 on the DNA sequence recognition by p50: p65 seems to cause an unequal interaction of the p50 molecules with the decameric cognate sequence. In one case, this is evident from the shift of the bending center towards the 3'-end of the motif, in the other case, by the preference of the heterotetramer for binding motifs of reduced symmetry and half sites of distinct sequence conservation. It is likely that these observations are directly related.

We assume that each half site of the xB motif is bound by one p50 molecule in a head to head orientation. The p50 molecules in the NF-xB homodimer appear to interact equally with the half sites of the xB motif 5'-GGGACTTTCC-3' with respect to the induction of DNA bending although the half sites are recognized with a slightly distinct affinity by the p50 molecules (9). The p50 molecules in the NF-xB heterotetramer recognize the half sites of the xB motif not only with different affinity but, in contrast to the dimer, the p50 molecules appear to subject their half sites differently to a structural modification. This raises the possibility that the two p50 molecules in the heterotetramer are modified by the p65 molecules such that they received distinct DNA-binding and -bending properties. It is however hard to envision how two indistinguishable p65 molecules in the heterotetramer can give raise to two distinct p50 molecules. An alternative possibility is that both p50 molecules are still functionally equivalent but are altered in their quaternity structure by p65. We indeed have evidence that the p50 molecules in the heterotetramer are differently linked than in the dimer (M.B. Urban and P.A. Baeuerle, in preparation). An alternative determinant for their differential interaction with the half sites of the xB motif would than be the distinct DNA sequences of the half sites. Half site B might preferentially undergo bending because it has a higher content of A and T bases. Certain A-T rich DNA stretches from kinetoplast DNA were shown to bend even in the absence of proteins (33). One p50 molecule would bind the more conserved 5'-half site A with high affinity, which serves to stabilize the protein-DNA interaction, while the second p50 molecule binds more weakly to the less conserved 3'-half site B but can alter its helical structure. Further studies are required to investigate the sequence of events leading to NF-xB-DNA recognition and to obtain a more highly resolved picture of the DNA structure induced by p50 in the absence and presence of the p65 subunit.

MATERIALS AND METHODS

Oligonucleotides and vectors

Oligonucleotides were synthesized on an Applied Biosystems synthesizer and purified on cartridges (Applied Biosystems) according to the instructions provided by the manufacturer.

An EcoRI-HindIII fragment from the pBend2 vector (23) was cloned into an EcoRI-HindIII cleaved pUC9 vector (34). pUC-xB-Bend resulted from the insertion of a 30 bp oligonucleotide (Fig. 3A) with Sail linker sites into the Sail opened pUC9-Bend. Fragments of identical length were excised from the plasmid by the indicated restriction endonucleases (all purchased from Biolabs).

Electrophoretic mobility shift assays

The conditions for the standard EMSA are described elsewhere (15,35). The oligonucleotide used as DNA probe in Fig. 1 was labeled with α-[32P]-dCTP by the Klenow polymerase (Boehringer).
Modifications of EMSA conditions that were applied with the circularly permuted DNA fragments are indicated in Table 1. Phosphatase-treated circularly permuted DNA fragments were end-labeled with polynucleotide kinase (Biolabs) as described (36) and purified on a polyacrylamide gel followed by electroelution. For one assay approximately 10,000 c.p.m. (Cerenkov counting; 4.5 fmole) were used. Two hundred nanograms of poly(dI-dC) (Pharmacia) were included per assay. The NF-xB proteins were purified as described earlier (7,8,15).

Hydroxyl radical missing contact analysis

A double-stranded 30 bp oligonucleotide with the NF-xB site 5'-GGGACTTTCC-3' and flanking sequences from the mouse x light chain enhancer (sequence in ref. 15) was cloned into the HindIII and SalI sites of the Bluescript minus vector (Stratagene) and 32P-labeled at the 5'-end by a strategy analogous to that described previously (37). Hydroxyl radical treatment of the labeled DNA was as follows: 650 fmole of DNA in 25 mM HEPES-KOH, pH 7.5, and 150 mM NaCl were incubated for various times with 10 mM ascorbic acid, 10 pM H2O2. Reactions were stopped by the addition of 10 mM thiourea, 2 mM EDTA and DNA was precipitated with linear poly aery lamide. The extent of the reaction was determined by electrophoretic analysis. The sample with a ratio of reacted to unreacted DNA of 3:10 was used for EMSA.

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