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The Histone Octamer Is Invisible When NF- κ B Binds to the Nucleosome*

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The transcription factor NF- κ B is involved in the transcriptional control of more than 150 genes, but the way it acts at the level of nucleosomal templates is not known. Here we report on a study examining the interaction of NF- κ B p50 with its DNA recognition sequence in a positioned nucleosome. We demonstrate that NF- κ B p50 was able to bind to the nucleosome with an apparent association constant close to that for free DNA. In agreement with this, the affinity of NF- κ B p50 binding does not depend on the localization of its recognition sequence relative to the nucleosome dyad axis. In addition, the binding of NF- κ B p50 does not induce eviction of histones and does not perturb the overall structure of the nucleosome. The NF- κ B p50-nucleosome complex exhibits, however, local structural alterations within the NF- κ B p50 recognition site. Importantly, these alterations were very similar to those found in the NF- κ B p50-DNA complex. Our data suggest that NF- κ B p50 can accommodate the distorted, bent DNA within the nucleosome. This peculiar property of NF- κ B p50 might have evolved to meet the requirements for its function as a central switch for stress responses.

The Rel/NF- κ B protein family regulates several vital processes in mammalian cells, including inflammation and immune responses, formation of dorsal-ventral polarity, cell adhesion, cancer, and apoptosis (1–4). The large role of these transcription factors in the cellular life cycle explains the high interest in their function. When bound to DNA the Rel/NF- κ B proteins are found either as homo- or heterodimers. The crystal structures of NF- κ B p50 and NF- κ B p52 homodimers bound to DNA have been solved previously (5–7). A detailed picture of

the structure and dynamics of these complexes in solution is, however, still missing.

The basic unit of chromatin, the nucleosome, is formed upon the wrapping of two superhelical turns of DNA around an octamer of core histones (two of each of H2A, H2B, H3, and H4). The structures of both the histone octamer and the nucleosome were solved by x-ray crystallography (8–10). Under physiological conditions the nucleosomal arrays fold into higher order structures. The chromatin higher order structures and the nucleosome internal architecture both might interfere with the binding of the transcription factors to their recognition DNA sequence. Two main strategies used by the cell to allow transcription factors to overcome the nucleosome-imposed structural impediments have been the focus of several studies. The first strategy uses ATP-dependent chromatin-remodeling activities. Several remodeling complexes have been isolated and studied (11–15), and all of these complexes are able to assist histone octamer sliding in an ATP-dependent manner and thus to facilitate access of nucleosomal DNA to transcription factors (for recent reviews, see Refs. 16 and 17). In the second strategy, a transient unraveling of a portion of the nucleosomal DNA is used as a key event in increasing the accessibility of the transcription factor recognition site (18–20). In this case a reduction in the association constant of the transcription factors in the order of 10^2 – 10^5 is expected, depending on the localization of the binding site relative to the nucleosome dyad (18).

In addition to these two strategies, the cell may also use specially designed transcription factors that are able to overcome the nucleosome barrier and to bind to nucleosomal DNA (21). Indeed, several transcription factors, including steroid hormone receptors, can bind efficiently to nucleosomal DNA (21–23). The mechanism of binding of these transcription factors as well as the nature of the perturbations of the nucleosome structure induced by their binding is, however, largely unknown.

Here we analyzed the binding of NF- κ B p50 to its recognition sequence in positioned nucleosomes. It is shown that at equilibrium, a dimer of NF- κ B p50 is found bound to nucleosomal DNA as efficiently as to naked DNA templates. In addition, the overall structure of the nucleosome within NF- κ B p50-nucleosome complexes was preserved. The local alterations in the NF- κ B p50 recognition site in the NF- κ B p50-nucleosome complexes were very similar to those found in the NF- κ B p50-naked DNA structures. Therefore, the presence of the core histones does not significantly interfere with the interaction of

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NF- κ B p50 with nucleosomal DNA. We hypothesized that this specific property of NF- κ B p50 is required for fulfilling its functions as a key factor for the different stress responses.

EXPERIMENTAL PROCEDURES

Oligonucleotides—37-Mer oligonucleotides 5'-ACGCACTCGAGTTG-GGGATTCCCAGTCTAGAGAGTG and 5'-CACTCTCTAGACTGGG-GAATCCCCAACTCGAGTACGT were purchased from Genosys (UK) and gel-purified. Typically 10 pmol from either the top or the bottom strand were 5'-end-labeled by T4 polynucleotide kinase with [γ - 32 P]ATP. The specific activity of the labeled probe was 2–3 \times 10⁶ cpm/pmol. After annealing with the complementary strand, DNA was treated by Fpg DNA N-glycosylase (a kind gift from Serge Boiteux, Commissariat à l'Énergie Atomique-Fontenay aux Roses) to remove preexisting oxidative guanine lesions, gel-repurified, and reannealed (31). Under the conditions used, 100% of duplexes were obtained.

DNA Fragments—Plasmid pXP10 was digested with EcoRI and RsaI, and the 152-bp fragment containing the *Xenopus borealis* somatic 5 S gene was isolated by standard procedures (44). The QuikChangeTM site-directed mutagenesis kit (Stratagene) was used to introduce the recognition sequence of NF- κ B p50 (ggggattcccc) at positions -16 to -26 (NF1), -41 to -51 (NF2), and -53 to -63 (NF3). The 5'- or 3'-ends at the EcoRI site of the 152-bp fragment were 32 P-labeled by using either polynucleotide kinase and [γ - 32 P]ATP or klenow fragment and [α - 32 P]ATP. The typical specific activity was 2–3 \times 10⁷ cpm/ μ g.

Nucleosome Reconstitution—Recombinant *Xenopus laevis* full-length histone proteins were used for nucleosome reconstitution (45). Nucleosome reconstitution was carried out as described previously (46). Briefly, a stoichiometric solution of the four histones in 10 mM HCl was dialyzed overnight at 4 °C against histone-folding buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 5 mM β -mercaptoethanol, 2 M NaCl). The next morning, histones were mixed at a 0.8/1 w/w ratio with 60 ng of the 152-bp labeled DNA probes and 550 ng of "bulk" nucleosomal DNA (prepared from native hen erythrocyte nucleosomes) in a final volume of 150 μ l of 1 \times histone-folding buffer containing 100 μ g/ml bovine serum albumin. The reconstitution was performed by dialysis against solutions containing decreasing concentrations of NaCl. Finally, the reconstituted particles were dialyzed against 10 mM Tris, pH 7.8, 10 mM NaCl, 1 mM EDTA. The protocol of Adams and Workman (47) was used to produce and purify recombinant human NF- κ B p50. The NF- κ B p50-DNA and NF- κ B p50-nucleosome complexes were formed according to Angelov *et al.* (22).

Electrophoretic Mobility Shift Assay and Hydroxyl Radical and DNase I Footprinting—The binding reactions were carried out in 10 μ l of solution of 20 mM Hepes, 100 mg/ml bovine serum albumin, 50 mM NaCl, and 5% glycerol, pH 7.5. Carrier nucleosomes were added to the naked NF1 and NF3 DNA samples to concentrations equal to those in the labeled nucleosome samples. 5–10 fmol of either labeled nucleosomes or free DNA were mixed with the indicated amounts of NF- κ B. After incubation for 15 min at 30 °C the samples were analyzed by electrophoretic mobility shift assay (EMSA).¹ The EMSA was done in a 4% acrylamide gel at room temperature in 0.5 \times or 0.25 \times Tris-borate-EDTA (TBE) buffer as described previously (46). The gels were then dried and exposed overnight on a PhosphorImager screen. For the analysis of the stoichiometry of NF- κ B p50-nucleosome interaction, the gel was incubated in 1 \times SDS-PAGE running buffer, and the proteins were transferred onto a nitrocellulose membrane. Western blot analysis was performed using the NF- κ B p50 antibody sc-1190 (Santa Cruz Biotechnology) and an Alexa anti-goat secondary antibody according to the manufacturer's recommendations. DNase I footprinting was performed essentially as described by Angelov *et al.* (22–24). The hydroxyl radical footprinting was carried out according to the protocol of Hayes and Lee (48).

UV Laser Footprinting—The NF- κ B p50-DNA complexes were formed (22), and both the resulting bound biomolecules and the naked oligonucleotide were exposed to either a single high intensity UV pulse ($E_{\text{pulse}} = E_{\text{total}} = 0.1$ J/cm²) or multiple low intensity UV pulses ($E_{\text{pulse}} = 0.01$ J/cm²; $E_{\text{total}} = 0.2$ J/cm²). The DNA was then purified by phenol-chloroform extraction, dissolved in an appropriate buffer, and treated with piperidine or digested with either Fpg protein or T4 endonuclease V (31, 34). Following lyophilization, DNA was resuspended in formamide loading buffer, heated briefly at 90 °C, and run on either 15 or 8% sequencing gel for the separation of the digested products of the 37- or 152-bp DNA fragments, respectively. The gels were dried, ex-

posed overnight on a PhosphorImager screen, and processed by using ImageQuant 4.1 software (Amersham Biosciences). The quantum efficiency of cleavage (relative cleavage yield/number of photons absorbed/base, assuming linear absorption approximation) was determined as described previously (34).

Electron Cryomicroscopy—The electron cryomicroscopy (EC-M) analysis was carried out on NF- κ B p50-NF1 nucleosome complexes. NF1 nucleosomes were reconstituted and brought to about 50 μ g/ml. Increasing amounts of NF- κ B p50 were added to a fixed amount of NF1 nucleosomes, and the complexes thus formed were analyzed by EMSA. The conditions that allowed the assembly of 50% of NF- κ B p50-NF1 nucleosome complexes were determined and used for EC-M. A 3- μ l drop of the sample solution prepared as described above was applied on an electron microscopy grid covered with perforated supporting film; the excess of solution was removed with blotting paper (Whatman 40), and the grid was projected by a gravity-driven plunger into liquid ethane (~110 K). Without rewarming, the grid was mounted in a cryoholder (Gatan, Pleasanton, CA) and transferred into a Philips CM200 microscope. Low dose images were recorded on Kodak SO163 film at 80 kV acceleration voltage at \times 66,000 direct magnification with an underfocus of 1.5 μ m. The images were scanned using a Charged Coupled Diodes camera with a resolution of 8.8 μ m/pixel, low pass-filtered (Gaussian mask with variable radius), and optimized in brightness and contrast.

RESULTS

NF- κ B Is Able to Bind to Its Recognition Sequence Inserted within a Positioned Nucleosome—To determine whether NF- κ B p50 was able to interact with positioned nucleosomes, three different constructs, each containing a single NF- κ B p50 binding site inserted at different locations within a 152-bp fragment comprising the 5 S RNA gene of *X. borealis*, were made. Because the *X. borealis* 5 S RNA gene contains a strong positioning signal, this allowed the reconstitution of precisely positioned nucleosomes (NF1, NF2, and NF3) containing the NF- κ B p50 target sequence located at different distances from the nucleosome dyad (Fig. 1A). Recombinant highly purified *X. laevis* histones were used for reconstitution of nucleosomes. EMSA of the reconstituted particles using low ionic strength conditions showed that complete reconstitution was achieved because no or very little free DNA was observed on the gel (Fig. 1B) and showed that all particles migrated with the same mobility, suggesting that there were no detectable alterations of the positioning of the histone octamer on the 5 S DNA fragment.

Proteins present in the gel-purified nucleosome particles were analyzed by SDS-PAGE (Fig. 1C). The expected protein composition for a nucleosome particle and the integrity of histones were preserved in the reconstituted nucleosomes. It should be noted that recombinant histones H2A and H2B show the same electrophoretic mobility and cannot be well separated on the SDS gel (Fig. 1C). To further characterize the reconstituted nucleosomes on these altered 5 S DNA sequences, a DNase I footprinting was performed on each reconstituted nucleosome (Fig. 1D). A clear footprint pattern in all three reconstituted nucleosomes, NF1, NF2, and NF3, identical to the pattern obtained on the 5 S nucleosomal template was observed, indicating that the introduction of the NF- κ B binding site in the 5 S DNA sequence does not perturb nucleosome formation and positioning.

To test whether NF- κ B p50 was able to interact with these nucleosomes, NF1, NF2, and NF3, we determined the apparent dissociation constant of NF- κ B p50 for nucleosomes and compared it with that of free DNA (Fig. 2A). Briefly, DNA of NF1, NF2, and NF3 was radioactively end-labeled and reconstituted into nucleosomes. Increasing amounts of NF- κ B p50 were incubated with identical amounts of NF1, NF2, or NF3 nucleosomes or with naked NF1 or NF2 DNA. The respective complexes were allowed to form, and they were then separated on native polyacrylamide gels. The portions of NF- κ B p50 un-

¹ The abbreviations used are: EMSA, electrophoretic mobility shift assay; EC-M, electron cryomicroscopy; 8-oxoGua, 8-oxo-7,8-dihydroguanine.

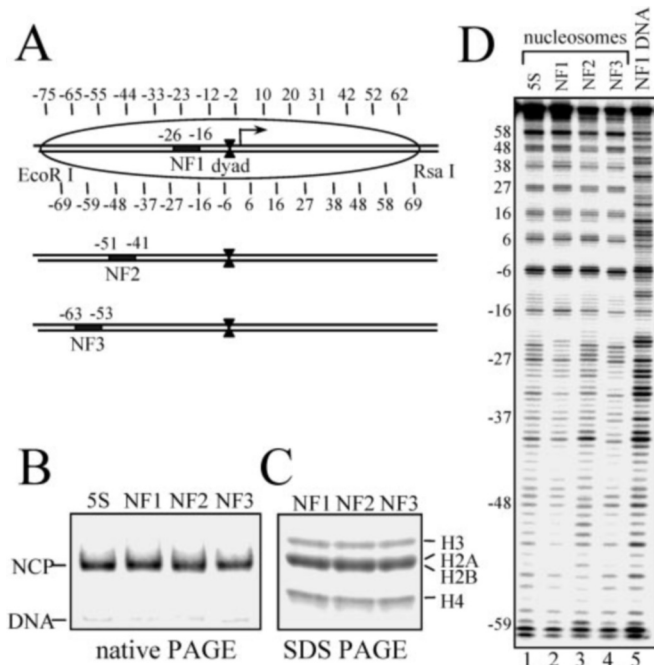


FIG. 1. Reconstitution of positioned nucleosomes with the 5 S DNA sequence containing the NF- κ B binding site. Three different constructs, NF1, NF2, and NF3, containing a single NF- κ B binding site within the 152-bp fragment comprising the *X. borealis* somatic 5 S RNA gene were built and used to reconstitute nucleosomes. **A**, schematic presentation of the reconstituted NF1, NF2, and NF3 nucleosomes. The location of the NF- κ B p50 binding sequence is shown in *bold*. The *triangles* show the dyad of the nucleosome, and the transcription site of the 5 S RNA gene is also designated. **B**, electrophoretic mobility shift assay of the three types of reconstituted nucleosomes compared with nucleosomes reconstituted with the wild type 5 S DNA sequence. The native gel was run in $0.25\times$ Tris-borate-EDTA. NCP, nucleosome core particle. **C**, 18% SDS-PAGE of histones isolated from reconstituted nucleosomes. **D**, DNase I footprinting analysis of the 5 S, NF1, NF2, and NF3 nucleosomes. The bottom strand of DNA was labeled at the 3'-end.

bound nucleosomes or NF- κ B p50 unbound free DNA at a given NF- κ B p50 concentration were calculated from the experimental data, and the dependences of unbound template *versus* added NF- κ B p50 were built (Fig. 2A). The apparent dissociation constants, corresponding to the values of the NF- κ B p50 concentrations at 50% unbound template, were thus determined. Remarkably, the apparent dissociation constants for the free NF1 and NF2 DNA and the NF1, NF2, and NF3 nucleosomes were very close: 5 nM *versus* 10 and 20 nM, respectively. Therefore, the presence of the nucleosomal histones interferes only slightly with the binding of NF- κ B p50 to its recognition sequence inserted within a nucleosomal DNA. To further characterize the interaction of NF- κ B p50 with the nucleosomal templates, we performed DNase I footprinting (Fig. 2B). Indeed, a clear footprint pattern corresponding to the interaction of NF- κ B p50 with its target site in all three reconstituted nucleosomes was observed (Fig. 2B, compare lanes 1, 3, and 5 with lanes 2, 4, and 6 for the top strand and lanes 1', 3', and 5' with lanes 2', 4', and 6' for the bottom strand). An increased sensitivity of DNA for DNase I digestion is observed close to the binding sites of NF- κ B p50 (positions -32 to -38), indicating that the binding of NF- κ B p50 induces local modification of DNA accessibility. Hence, NF- κ B p50 is able to bind to its cognate sequence inserted within nucleosomal DNA independently of its location relative to the dyad axis of the nucleosome. Interestingly, the DNase I-characteristic 10-bp digestion pattern of the nucleosomal structure is still clearly visible when NF- κ B p50 is bound, indicating that the overall nucleosomal structure is conserved. This conclusion is further supported by the EC-M data (Fig. 3).

EC-M has the advantage that it provides a relatively high resolution snapshot of the solution conformation of unfixed and unstained specimen. It was extremely powerful in studying both the structure of the nucleosome and the nucleosomal conformational transitions occurring upon the passage of SP6 RNA polymerase (25, 26). We used a 208-bp DNA fragment containing the 5 S RNA gene of *X. borealis* with the inserted NF- κ B p50 recognition sequence at positions -16 to -26 to reconstitute a nucleosome with the NF- κ B p50 recognition sequence located in the vicinity of the dyad. About 30 bp of free DNA are present on each end of this nucleosome, which allows easier visualization of nucleosomal DNA (Fig. 3). NF- κ B p50 was allowed to interact with both free DNA and the reconstituted nucleosome. The assembled complexes were visualized by EC-M (Fig. 3). The electron cryomicrographs of the NF- κ B p50-DNA complexes (Fig. 3A) clearly show the presence of a dense structure located roughly in the middle of the DNA molecule where the NF- κ B p50 recognition sequence was inserted. Such structures are not observed in the naked DNA fragment alone (Fig. 3B). We attributed this structure to the presence of bound NF- κ B p50. This dense structure was also observed in the micrographs of the NF- κ B p50-nucleosome complexes (Fig. 3, *d-f* and *d'-f'*), and it was absent in those of the nucleosome alone (Fig. 3, *a-c* and *a'-c'*). The dense structure was visualized in the middle of the nucleosome, as expected for a bona fide NF- κ B p50-nucleosome complex. Therefore, EC-M distinctly detected NF- κ B p50 bound to its recognition sequence within a positioned 5 S nucleosome. Interestingly, the overall shape of the nucleosomal DNA of the NF- κ B p50-nucleosome complexes was essentially the same as that of the nucleosome alone (Fig. 3, compare *a-c* with *d-f*) suggesting that the binding of NF- κ B p50 does not perturb the overall structure of the nucleosome or that the structural changes are minor and below the resolving power of EC-M imaging.

The NF- κ B-Nucleosome Complex Exhibits Both Histone Content and a Hydroxyl Radical Footprinting Pattern Identical to Those of the Nucleosome Alone—To further characterize the organization of DNA of the NF- κ B p50-nucleosome complex we used a hydroxyl radical footprinting analysis. Because of its small size and lack of sequence selectivity, this probe allows a detailed resolution of the DNA conformation within the nucleosome (27, 28). A NF1 nucleosome was incubated with NF- κ B p50; then the DNase I and hydroxyl radical footprinting was performed with (Fig. 4A, lanes 1, 2, 4, and 5) and without (lane 3) gel purification of the complex. The DNase I footprinting shows that NF- κ B p50 binds to its cognate sequence under the conditions used (Fig. 4A, lanes 1–2). The hydroxyl radical footprinting pattern of the NF- κ B p50 bound samples is the same as that of the nucleosome alone (Fig. 4A, compare lane 5 with lane 4). Indeed, in all cases a clear enhancement of the hydroxyl radical cleavage at every 10 bp is observed, a pattern typical for the nucleosome (27, 29). Interestingly, the hydroxyl radical pattern of the region, which is covered by the protein, is not perturbed. Therefore, the binding of NF- κ B is unlikely to affect the regular exposure of the nucleosomal DNA strands toward the solution. This conclusion is supported by the DNase I footprinting data of the NF- κ B p50-nucleosome complexes where the sequences flanking the NF- κ B p50 binding site exhibit a 10-bp cleavage repeat typical for the nucleosomes (Fig. 4A, lanes 1–2).

The interaction of a DNA-binding protein with the nucleosome may be associated with a removal of some of the core histones. For example, the loss of the H2A-H2B dimer was reported upon passage of RNA polymerase II through a nucleosome (30). If the above results and conclusions are correct, *i.e.*

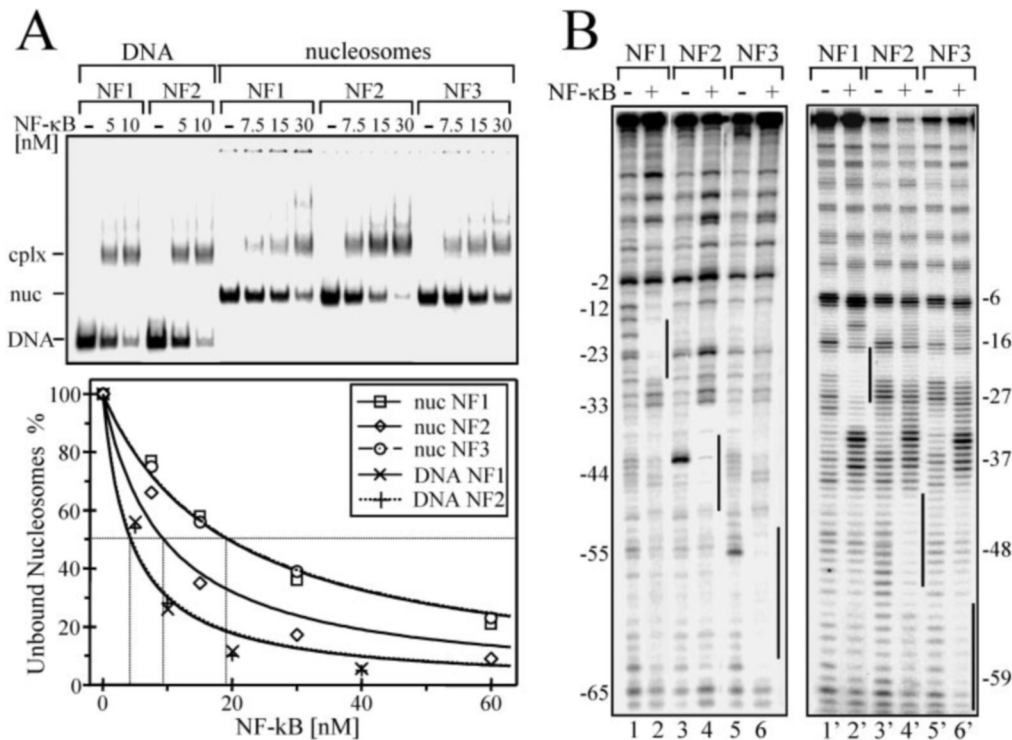


FIG. 2. NF- κ B-p50 exhibits very similar binding affinity for both free DNA and nucleosomes. *A*, typical EMSA of NF- κ B p50-DNA and NF- κ B p50-nucleosome complexes. An increasing amount of NF- κ B p50 was allowed to bind to NF1, NF2, or NF3 nucleosomes as well as to the respective naked NF1 and NF2 DNA fragments. The nucleoprotein complexes were then separated on a native 4% polyacrylamide gel. The positions of the naked DNA, the nucleosomes, and the NF- κ B p50-DNA and NF- κ B p50-nucleosome complexes are indicated. For the determination of the NF- κ B apparent dissociation constants K_d for DNA and for nucleosomes, the percentage of NF- κ B p50 unbound nucleosomes or NF- κ B p50 unbound free DNA at a given NF- κ B p50 concentration was calculated from EMSA data. Data points, averaged over at least three independent experiments, were fitted by non-linear least squares to the following binding equation: fraction unbound = $1/(1 + [\text{NF-}\kappa\text{B p50}]/K_d)$. The value of 50% unbound NF- κ B p50 to the nucleosome corresponds to the apparent constant of dissociation. Note that the apparent dissociation constant of NF- κ B p50 for free NF1 or NF2 DNA is only 2- and 4-fold less than for the NF2 nucleosome and the NF1 and NF3 nucleosomes, respectively. *B*, NF- κ B p50 was allowed to interact with the reconstituted nucleosomes, and its binding was visualized by DNase I footprinting. The recognition sequence of NF- κ B is designated by the vertical bold line. The top strand (lanes 1–6) was labeled at the 5'-end, and the bottom strand (lanes 1'–6') was labeled at the 3'-end.

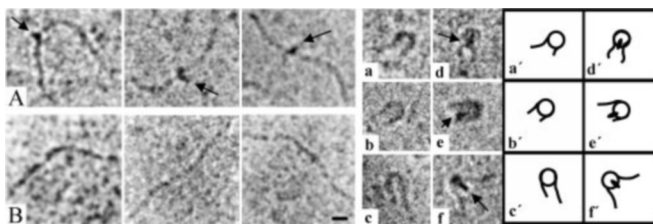


FIG. 3. Cryoelectron micrographs of DNA-NF- κ B p50 and nucleosome-NF- κ B p50 complexes. *A*, 205-bp DNA fragment containing the *X. borealis* somatic 5 S RNA gene with the inserted NF- κ B binding site at positions –16 to –26 was used to reconstitute a nucleosome. This nucleosome contains about 30 bp of free DNA on both of its ends, allowing easier visualization. *A*, cryoelectron micrographs for three 205-bp DNA-NF- κ B p50 complexes. The binding of NF- κ B p50 to DNA resulted in the generation of a dense structure (arrows) located roughly at the middle of the DNA filament. *B*, cryoelectron micrographs for three naked 205-bp DNA fragments. *a–c*, cryoelectron micrographs of three reconstituted nucleosomes. Note the free DNA ends of the nucleosomes. *d–f*, cryoelectron micrographs of three nucleosome-NF- κ B p50 complexes. The arrows designate NF- κ B p50 bound to nucleosomal DNA in the vicinity of the dyad axis. *a'–c'* and *d'–f'*, schematic drawings of the nucleosomes visualized on the *a–c* and *d–f* electron cryomicrographs, respectively.

the overall structure of the nucleosome is not affected by the binding of NF- κ B p50, one should expect that the binding of NF- κ B p50 would not be associated with the displacement of histones from the nucleosome. To test this we analyzed the protein composition of the complex formed when NF- κ B p50 is bound to nucleosomes (Fig. 4*B*). Briefly, NF1 nucleosomes (Fig. 4*B*, lane 1) and NF- κ B p50-NF1 nucleosome complexes (lane 2)

were separated on native polyacrylamide gel. The bands containing the nucleosomes (Fig. 4*B*, *nuc*, lane 1) or the shifted complex (*cplx*, lane 2) were cut from the gel, and then the proteins were eluted and analyzed on a SDS-polyacrylamide gel. Fig. 4*C* demonstrates that all four histones are present in equimolar amounts in both the nucleosomes alone and the NF- κ B p50-NF1 nucleosome complexes isolated from the gel (compare lanes 2 and 3). We conclude that the binding of NF- κ B p50 does not affect the histone content of the nucleosome.

A NF- κ B p50 Dimer Interacts with Nucleosomal DNA—The interactions of NF- κ B p50 with free DNA have been well studied and show that the protein is found bound on the DNA as a dimer, each monomer recognizing an 11-bp binding site in the major groove, and on opposite faces of the DNA helix. Because this mode of binding should be reconciled with nucleosomal DNA structure, it was important to define the stoichiometry of NF- κ B binding to positioned nucleosomal DNA. To this end the NF1, NF2, and NF3 DNA templates were ^{32}P end-labeled and then used to reconstitute nucleosomes. NF- κ B p50 was allowed to interact with both free NF1 DNA and the reconstituted nucleosomes, and then the complexes were analyzed on a native polyacrylamide gel (Fig. 5*A*). Interaction of NF- κ B p50 with the free NF1 DNA gives rise to a major shift (Fig. 5*A*, *cplx1*) corresponding to the interaction of a dimer with the binding site (see also Fig. 2). In presence of an excess of NF- κ B p50 a slightly slower migrating band (Fig. 5*A*, *cplx2*), corresponding to higher order complexes containing additional non-specifically bound NF- κ B p50, is also observed. In the presence of the nucleosomal templates NF1, NF2, and NF3, similar

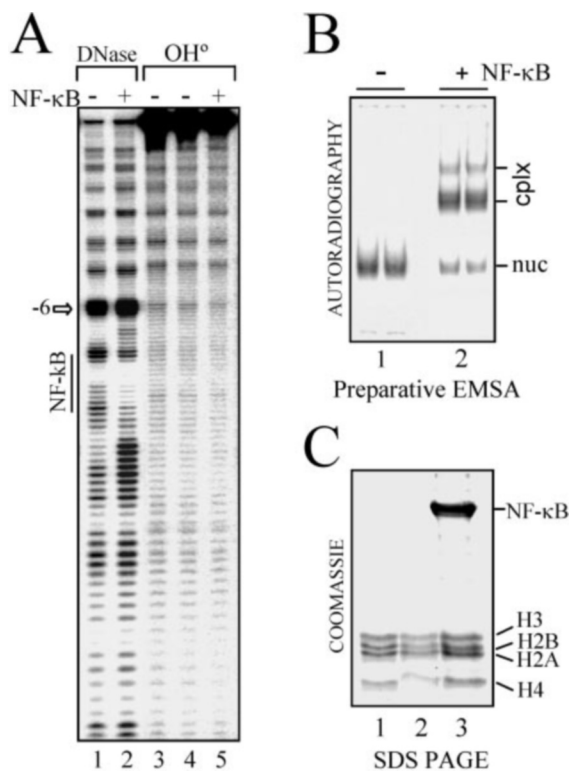


FIG. 4. The binding of NF- κ B p50 does not affect either the histone composition of the nucleosome or the exposure of nucleosomal DNA toward the solution. *A*, DNase I (lanes 1–2) and hydroxyl radical (OH \cdot) (lanes 3–5) footprinting of NF1 nucleosome-NF- κ B p50 complexes. The bottom DNA strand was labeled at its 3'-end. The vertical line and the arrow show the binding site of NF- κ B p50 and the cleavage position at -6 nucleotides, respectively. Lanes 1, 2, 4, and 5 are from complexes purified from native gels, whereas in lane 3, nucleosomes subjected to hydroxyl radical footprinting were not gel-purified. Note that the binding of NF- κ B p50 to the nucleosome does not perturb the hydroxyl radical footprinting. *B*, preparative gel shift used for analysis of the histone composition of the NF- κ B p50-nucleosome complexes. NF1 nucleosomes (1, the first two lanes) and NF1 nucleosome-NF- κ B p50 complexes (2, the second two lanes) were separated on a preparative native 4% polyacrylamide gel; the bands containing the respective nucleoproteins were eluted, and the protein composition was analyzed by 18% SDS-PAGE. *C*, protein composition of NF1 nucleosomes (lane 1) and eluted from the gel NF1 nucleosomes (lane 2) and NF1 nucleosome-NF- κ B p50 complexes (lane 3). The bands corresponding to the core histones and NF- κ B p50 protein are indicated. Note the identical histone composition of the samples. In this particular experiment, histones isolated from chicken erythrocytes were used for nucleosome reconstitution, which allowed a good resolution of the bands corresponding to H2A and H2B, respectively.

complexes (but with a slightly lower mobility) were observed (Fig. 5A, lanes 2–4). To determine the stoichiometry of the interaction of NF- κ B p50 with nucleosomal DNA compared with the interaction with free DNA, the complexes resolved on the native polyacrylamide gel were transferred onto a nitrocellulose membrane, which was used to perform a Western blot analysis with an antibody against NF- κ B (Fig. 5B). This Western blot was revealed using an Alexa anti-goat secondary antibody. The quantification and normalization of these two sets of data are shown in Fig. 5C. Remarkably, the amount of fluorescence corresponding to bound NF- κ B p50 normalized to the amount of nucleosomal DNA is identical to what is observed for the free NF1 DNA for both complex 1 (Fig. 5C, *cplx1*) and complex 2 (*cplx2*) indicating that the stoichiometry of the interaction is the same for the free and the nucleosomal DNA. Because NF- κ B interacts with the free DNA as a dimer, these data indicate that a dimer of NF- κ B p50 is able to somehow invade the nucleosomal particle.

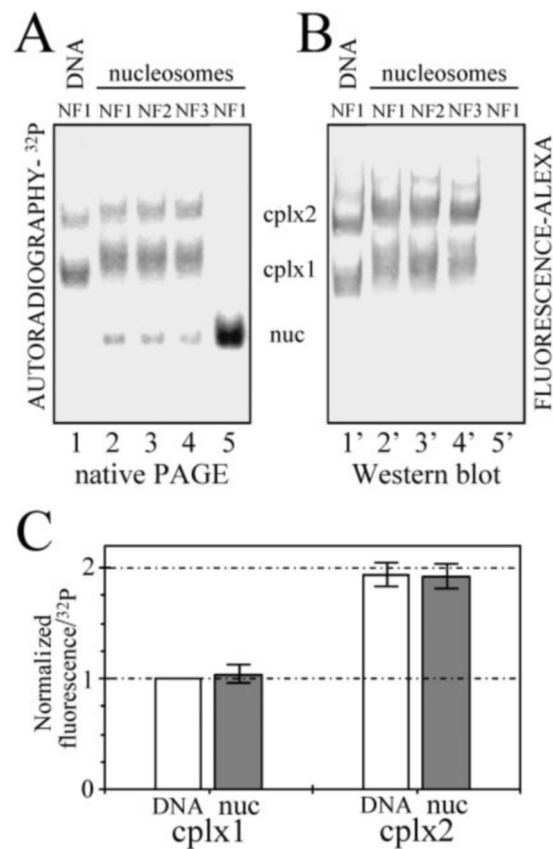


FIG. 5. Stoichiometry of the interaction of NF- κ B p50 with the nucleosomal DNA. *A*, NF- κ B p50 was allowed to bind to 32 P-labeled free NF1 DNA or to labeled NF1, NF2, or NF3 nucleosomes, and then the samples were separated on a native 5% polyacrylamide gel. The nucleoprotein complexes were exposed on a PhosphorImager screen. In lane 1 is shown the NF- κ B p50-DNA complex, whereas lanes 2–4 show the NF- κ B p50 complexed with NF1, NF2, or NF3 nucleosomes. In lane 5 is shown the migration of a free NF1 nucleosome. A small excess of NF- κ B was used to obtain full shift of free DNA and of nucleosomes. The positions of the unbound nucleosome (*nuc*) and the NF- κ B p50-DNA and NF- κ B p50-nucleosome complexes (*cplx1* and *cplx2*) are indicated. *B*, the proteins of the native gel shown in *A* were transferred to a membrane and then subjected to a Western blot analysis using an antibody against NF- κ B (sc-1190, Santa Cruz Biotechnology). The signals were quantified using PhosphorImager scanning. *C*, quantification of the amount of fluorescence (which detects the NF- κ B protein) normalized to the amount of labeled DNA for each complex (*cplx1* and *cplx2*). The presented data are averaged over three independent experiments.

The Binding of NF- κ B p50 to Nucleosomes Induces Structural Alterations in Nucleosomal DNA Identical to Those in Naked DNA Templates—We have shown above that NF- κ B p50 is able to bind to its recognition sequence inserted within nucleosomal DNA. This binding does not seem to affect the overall structure of the nucleosome and the wrapping of nucleosomal DNA around the histone octamer, and it is not associated with eviction of core histones. The x-ray studies of NF- κ B-DNA complexes showed, however, specific local deformation of the DNA structure of the recognition sequence (5–7). The question arises as to whether similar local changes of the NF- κ B recognition sequence are present within the NF- κ B p50-nucleosome complexes. To address this question we used the recently developed UV laser footprinting technique (31, 32).

The UV laser footprinting is based on exposure of the DNA to high intensity laser pulse(s), which results in the induction of specific photolesions that arise via biphotonic excitation of the nucleobases (33). The photodamage formation depends on both the DNA sequence and its structure (34). The binding of a transcription factor induces a local change in the DNA struc-

ture at the binding site, which in turn affects the type and spectrum of the lesions induced upon laser irradiation. Because the DNA base photomodifications can be mapped at the nucleotide level (31, 32, 34), this allows the visualization of local changes in DNA conformation at a nucleotide resolution, *i.e.* to carry out a UV laser footprinting (31, 32).

Initially, we studied by UV laser footprinting the solution structure of the NF- κ B p50 homodimer complexed with a 37-bp DNA duplex that contained the major histocompatibility complex H2 NF- κ B p50 binding site (5'-GGGGATTCCCC). The complex was exposed to a single high intensity 266 nm laser pulse or multiple low intensity pulses. A treatment with hot piperidine or digestion with either Fpg or phage T4 endonuclease V allowed the visualization of the changes in the nucleobase photoreactivity upon binding of the transcription factor. The results showed a clear footprint within the DNA binding sequence (Fig. 6, A and B).

The quantitative analysis of the laser photolysis-mediated one-electron oxidation nucleobase lesions revealed interesting structural alterations concerning the NF- κ B p50 DNA binding site within the NF- κ B p50-DNA complex (Fig. 6C). Indeed, the quantum efficiency for 8-oxoGua formation decreases 11–13 \times for the two central residues G-3 and G-4 in the guanine run of the bottom strand and 4 \times for the related nucleobases G+3 and G+4 in the top strand (Fig. 6C, *Fpg, High I*) compared with the naked oligonucleotide. It may be added that a decrease to a smaller extent in 8-oxoGua induction is observed in the first guanine residue (G+5 or G-5) of each individual strand (Fig. 6C, *Fpg, High I*). In contrast, only a small decrease in the quantum efficiency for 2,2,4-triamino-5-(2H)-oxazolone (Oz) (35) generation is noticed for these three guanines upon NF- κ B p50 binding (Fig. 6C, *pip, High I*). The picture is, however, different for the guanine G+2 (or G-2), for which an increase in the induction quantum efficiency is observed for both 8-oxoGua and Oz (Fig. 6C, *Fpg, High I* and *pip, High I*). The formation of cyclobutane pyrimidine dimers (36) and the pyrimidine (6-4) pyrimidone (PyrPyo) adducts within the NF- κ B p50 target site upon low intensity UV irradiation is markedly decreased in the presence of bound NF- κ B p50 (Fig. 6C, *Low I*). Hence, the analysis of the mono- and biphotonic DNA modifications provides insights into DNA alterations at the nucleotide level within the NF- κ B p50-DNA complex and visualizes a specific structural "signature" on DNA induced upon NF- κ B binding. This allows the use of UV laser light as a very sensitive tool for probing the structural perturbation of nucleosomal DNA induced upon binding of this transcription factor to positioned NF1, NF2, and NF3 nucleosomes (see Fig. 1).

Interestingly, the UV laser footprinting patterns of naked NF1, NF2, and NF3 DNA were identical to the nucleosomally organized DNA (Fig. 7, compare *lane 2* with *lane 6* and *lane 4* with *lane 8* in each panel). Therefore, the quantum efficiency of induction of monophotonic and biphotonic lesions is not affected by the wrapping of DNA around the histone octamer; *i.e.* the laser UV light does not "see" the wrapping of nucleosomal DNA, a result in agreement with the UV light genomic footprinting data (37). The binding of NF- κ B p50 to the naked 152-bp DNA results in the same structural alterations in the NF- κ B binding site as in the 37-bp duplex oligonucleotide (compare Fig. 7 with Fig. 6). Importantly, the specific NF- κ B p50 signature of DNA perturbation (a dramatic decrease of the quantum efficiency of 8-oxoGua in the first three residues of the guanine run (shown by *stars* on Fig. 7)) and a strong increase of the fourth G residue as well as the marked decrease in the yield of cyclobutane pyrimidine dimers (shown by *circles* on Fig. 7) detected upon low intensity UV irradiation were also observed (Fig. 7, compare *lane 2* with *lane 3* and *lane 4* with

lane 5 in each panel). Remarkably, the binding of NF- κ B p50 to the NF1, NF2, and NF3 nucleosomes results in the same structural alterations in the NF- κ B binding site as those in naked DNA (Fig. 7, compare *lanes 3* and *7* and *lanes 5* and *9* in each panel). Therefore, NF- κ B p50 can accommodate the bent nucleosomal DNA and can bind to its recognition sequence independently of its localization relative to the dyad.

DISCUSSION

We have studied the binding of NF- κ B p50 to its recognition sequence in positioned nucleosomes using DNase I, hydroxyl radical and UV laser footprinting analysis, EMSA, and electron cryomicroscopy. The combination of these methods allowed us to obtain information that was inaccessible to date. Our results demonstrate that the NF- κ B p50 recognition sequences within the nucleosomal DNA are accessible to NF- κ B p50. The stoichiometry of the interaction of NF- κ B p50 with nucleosomal DNA templates is identical to that of the interaction with free DNA. Interestingly, the binding of NF- κ B p50 did not alter the global structure of the nucleosome; the electron cryomicrographs show no difference in the overall structure of free nucleosomes and that of NF- κ B p50 bound particles. The 10-bp nucleosome-specific DNase I and hydroxyl radical footprinting patterns were not affected by the presence of NF- κ B p50, a result supporting the electron cryomicroscopy data. In agreement with this, it was illustrated that no eviction of histones was occurring upon NF- κ B p50 binding to the nucleosome. As demonstrated by the high resolution UV laser footprinting, the binding of NF- κ B p50 to the nucleosome results, however, in local alterations in the structure of its recognition site. Remarkably, these alterations were found to be very similar to those observed in the NF- κ B p50 DNA complex. Finally, the binding affinity of NF- κ B p50 to both free DNA and nucleosomally organized templates was found to differ only very slightly. All these data allow us to conclude that the histone octamer does not significantly affect the binding of a homodimer of NF- κ B p50 to the nucleosome; *i.e.* the histone octamer is "transparent" with respect to the binding of NF- κ B p50 to the nucleosome. This implies that NF- κ B p50 has evolved to be compatible with the histone octamer-DNA interactions.

Why does NF- κ B exhibit such a peculiar property? Could this be related to its function as a central regulator of stress responses? (For review, see Ref. 4.) Indeed, in most cells the Rel/NF- κ B proteins are found in the cytoplasm under the form of inactive complexes with their inhibitor I κ B. Numerous stimuli are able to induce an activation of these transcription complexes by freeing them from I κ B (4). The Rel/NF- κ B proteins are then translocated into the nucleus where they bind to their recognition DNA sequence and activate transcription. The stress response requires rapidity and high efficiency. To fulfill these requirements it appears that NF- κ B proteins developed a very interesting property, a compatibility with histone-DNA interactions. This property of NF- κ B could explain why eukaryotic cells respond to so many different stimuli by activating the same transcription factor. Indeed, the currently favored model for gene activation suggests that gene-specific activators directly recruit histone acetyltransferase complexes or chromatin-remodeling machines to the promoters of target genes (38–42). This activator-dependent recruitment supposes, however, that the activator can easily invade the nucleosome and bind to its recognition sequence. NF- κ B p50 possesses this property, which in turn suggests that it might be able to interact with different histone acetyltransferases and chromatin-remodeling machines and to recruit them to the target genes. Subsequently, this would allow chromatin to be remodeled and transcription of the NF- κ B target genes to be activated.

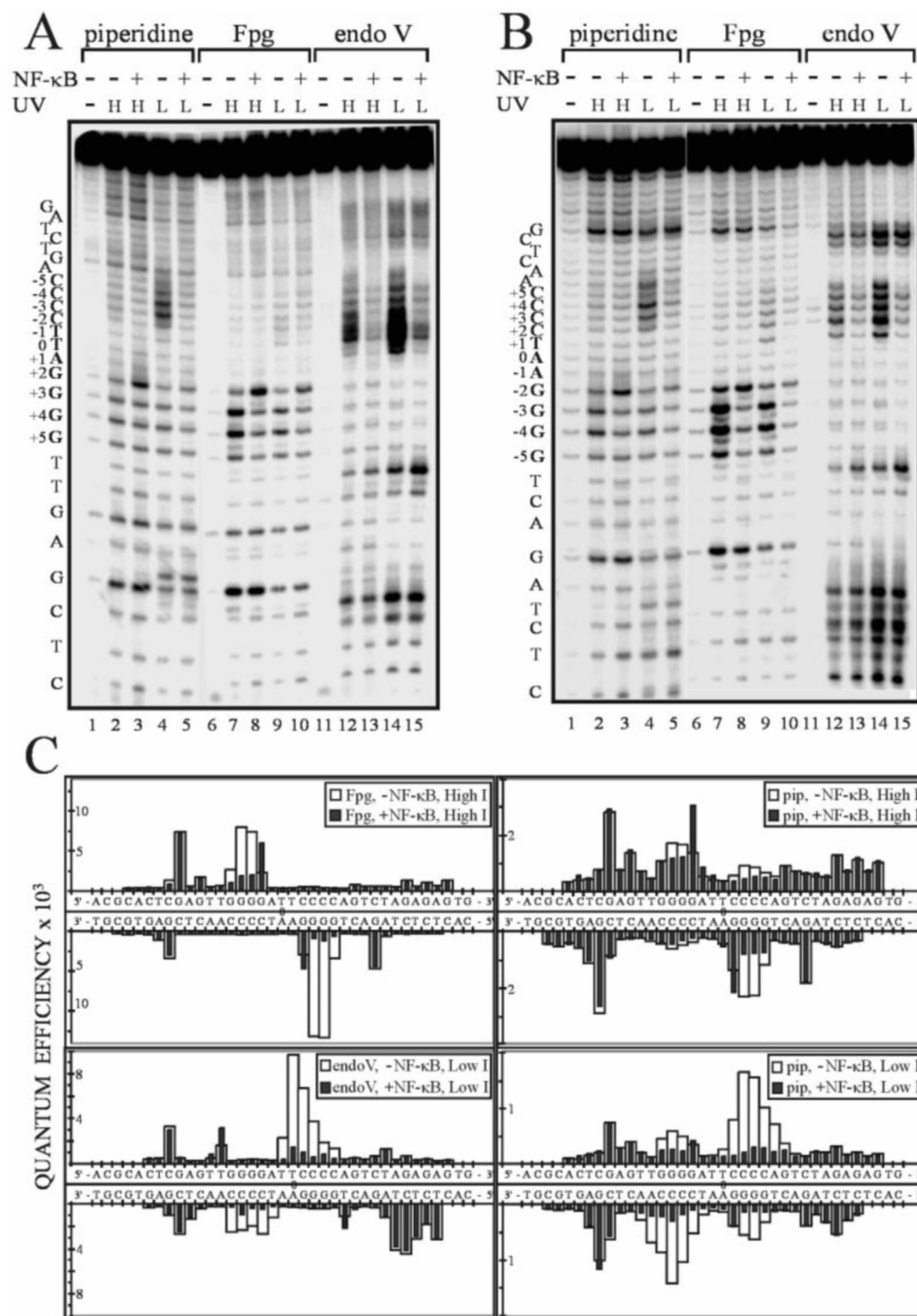


FIG. 6. UV laser footprinting of NF- κ B p50 homodimer-DNA complexes. A 37-mer double-stranded oligonucleotide containing the binding sequence of NF- κ B p50 was allowed to interact with NF- κ B p50. EMSA was used to find the conditions for complete (100%) binding of NF- κ B p50, and the footprinting analysis was carried out. A, UV laser footprinting of the NF- κ B p50 homodimer-DNA complexes on the top strand. The 5'-end of the top strand of the oligonucleotide was labeled with γ - 32 P. The NF- κ B p50-DNA complex was formed and exposed to a single high intensity (H) UV laser pulse ($E_{\text{pulse}} = 0.1 \text{ J/cm}^2$) or multiple low intensity (L) pulses ($E_{\text{pulse}} = 0.01 \text{ J/cm}^2$; $E_{\text{total}} = 0.2 \text{ J/cm}^2$). Then the samples were treated with piperidine or digested with either Fpg or T4 endonuclease V, and the products of digestion were separated on a 15% polyacrylamide sequencing gel. On the left of the panel are indicated the positions of the cleaved base corresponding to the respective fragments. The NF- κ B p50 binding sequence is in bold. B, UV laser footprinting of the NF- κ B p50 homodimer-DNA complexes on the bottom strand. C, quantification of the data shown in A and B. The quantum efficiency of the formation of the different lesions induced at high intensity (High I) or low intensity (Low I) in the absence of NF- κ B p50 (-NF- κ B) or in its presence (+NF- κ B) is reported.

The affinity of NF- κ B p50 for a nucleosome does not depend on the localization of its recognition site relative to the dyad axis (at least for the sites tested here), and it only slightly differs from the affinity for naked DNA templates. Our data show that the binding of NF- κ B p50 to nucleosomes is associated with a slight increase of DNase I sensitivity of DNA

sequences around -37 (see Fig. 2), suggesting that the conformation of this DNA region of the nucleosome has been altered by the binding of NF- κ B p50. Disruption of a few histone-DNA contacts within this region might be sufficient for the binding of NF- κ B p50 dimer. At this point our data cannot be easily reconciled with the crystal structure of the nucleosome (10) and

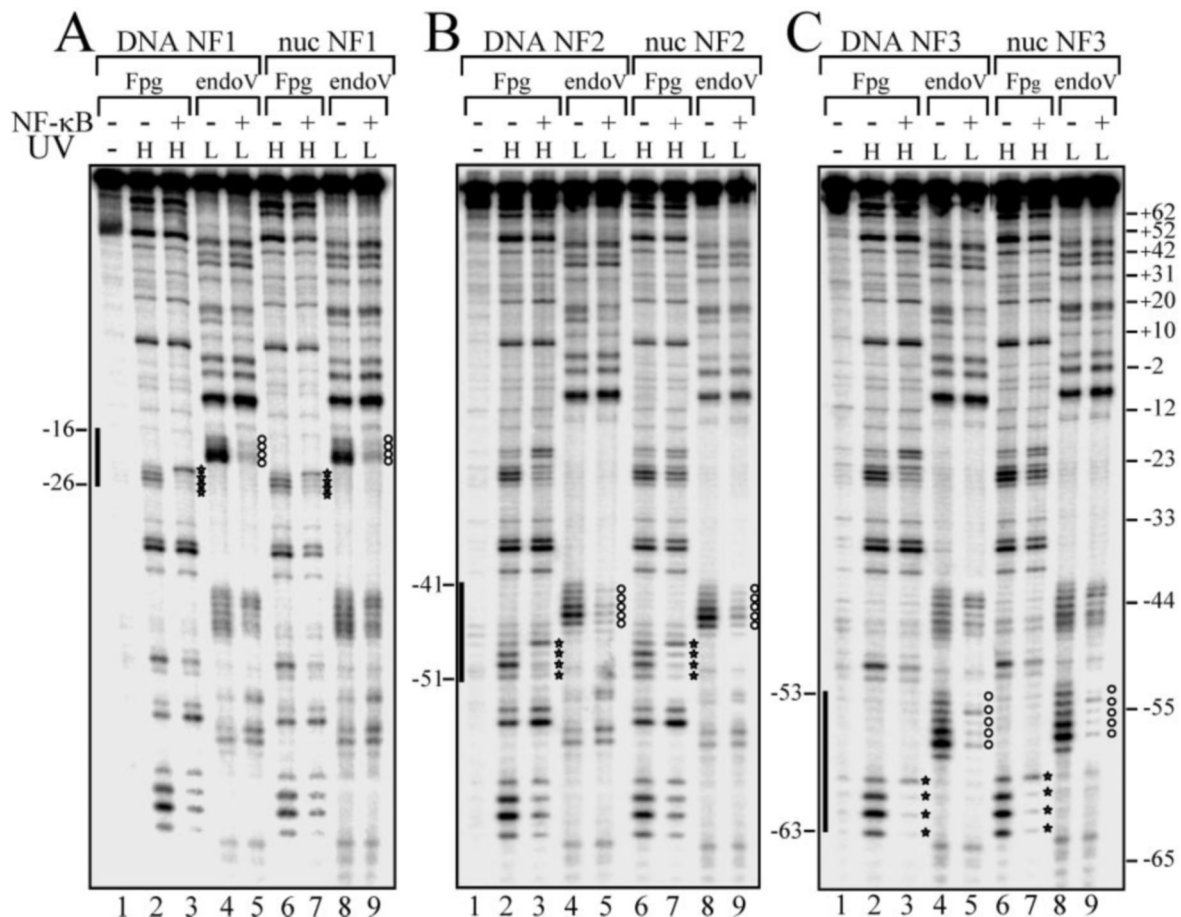


FIG. 7. The UV laser footprinting shows that the binding of NF- κ B p50 to nucleosomes induces structural alterations in nucleosomal DNA identical to those induced in naked nucleosomal DNA. The 5'-end of NF1, NF2, and NF3 DNA was labeled with γ - 32 P and used for reconstitution of nucleosomes. The complexes of NF- κ B p50 with NF1, NF2, and NF3 nucleosomes and NF1, NF2, and NF3 naked DNA were irradiated with a single high intensity (H) UV laser pulse or multiple low intensity (L) pulses. Then the samples were treated with Fpg or T4 endonuclease V, respectively, and the products of digestion were separated on a polyacrylamide sequencing gel. The vertical lines designate the location of the NF- κ B p50 recognition sequence. A, UV laser footprinting of NF- κ B p50 complexes with NF1 DNA and NF1 nucleosomes. B and C, UV laser footprinting of NF- κ B p50 complexes with NF2 and NF3 DNA and NF2 and NF3 nucleosomes, respectively.

NF- κ B-DNA complexes (5–7). NF- κ B transcription factors almost completely enclosed their DNA target sites, and docking NF- κ B onto the nucleosome should lead to steric clashes with the histone octamer bound to the nucleosomal DNA. Integration of these currently disparate data will require more detailed analysis taking into account the dynamic properties of all components. It is possible that only one p50 monomer binds first to nucleosomal DNA and that the p50-cognate sequence is then peeled off away from the surface of the histone octamer, which allows the binding of the second p50 monomer.

Several transcription factors including Sp1, LEF-1, and upstream stimulatory factor (22, 23) as well as some steroid hormone receptors can bind to their cognate sites within the nucleosome (for review, see Ref. 21). It is possible that at least some of these factors might exhibit like NF- κ B a compatibility with the histone octamer-DNA interactions; *i.e.* the histone octamer might be “compliant” when these transcription factors bind to the nucleosome. We hypothesize that many other DNA-binding proteins, including enzymes, also may have developed DNA binding properties compatible with the presence of the histone octamer within the nucleosome. The histone-DNA interaction compatibility of these proteins would greatly help their function on nucleosomal templates. In support of this are the recent data that illustrate that human DNA ligase I seals nicks in nucleosomal DNA with high efficiency (43).

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