NF-κB is an inducible transcription factor involved in the immune response, inflammation, and viral transcription. To address how the two NF-κB and three Sp1 binding sites of the human immunodeficiency virus (HIV) long terminal repeat (LTR) control multiple activator assembly and transcription, we first observed and compared unique conformations between the crystallographic structure of the NF-κB p50p65 heterodimer bound to the uPA-κB target site to that of the p50p65 HIV-κB complex. Next, cooperativity between two NF-κB molecules bound to tandem HIV-κB sequences was measured as well as that of NF-κB and transcription factor Sp1 when bound to adjacent sites. The cooperativity of hybrid HIV-LTR enhancers was measured with the 3′ κB site converted to uPA-κB or to interferon β gene enhancer κB. The hybrids were defective in transcriptional activator assembly and less actively transcriptionally. These functional differences correlate with observed conformational differences and demonstrate that distinct κB DNA sequences function as allosteric regulators in a gene-specific manner.

The mammalian Rel/NF-κB family polypeptides, p50, p52, c-Rel, p65 (RelA), and RelB, form transcriptionally active dimers in a combinatorial manner. Of 15 potential NF-κB homomers and heterodimers, the NF-κB p50p65 heterodimer is most prevalent in cells (1). Rel/NF-κB family members share an N-terminal Rel homology region (RHR) that is responsible for dimer formation and DNA binding to decameric κB sites. The x-ray crystal structure of the NF-κB p50p65 RHR heterodimer bound to the κB binding site of immunoglobulin κ light chain gene enhancer (Ig-κB) has been determined (2). The Ig-κB DNA sequence is identical to both κB sites contained within the long terminal repeat of the human immunodeficiency virus promoter (HIV LTR) and is therefore also referred to as HIV-κB. The x-ray structures of several other DNA-bound NF-κB homodimers reveal that the NF-κB RHR displays different conformations on various κB sites (3–6). Variability in NF-κB structure is accommodated by the bimodular architecture of the RHR; the N-terminal DNA binding domain (NTD) and C-terminal dimerization domain are connected by a short, flexible linker.

Most eukaryotic genes are regulated by a gene-specific enhanceosome consisting of a regulatory enhancer and its multiple bound activators. Through interactions with other factors on gene enhancers, NF-κB p50p65 heterodimer regulates hundreds of genes (1, 7). The sequence and dynamics of the gene-specific κB sites have been observed to affect NF-κB binding affinity in solution (8–10). It has been proposed that each of these κB site-specific NF-κB conformations is essential for the formation of a gene-specific enhanceosome.

The role of DNA as an allosteric effector is not a new one. Experiments in protein transactivation have led to an evolution in the view of transcription away from one in which a regulator is composed of two separable domains, a DNA binding domain that localizes a transactivation domain to the appropriate environment, to the newer paradigm in which DNA binding has direct effects on transactivation. One family of proteins in which the allosteric role of DNA is particularly well documented is the nuclear receptor family, which includes the subset of steroid hormone receptors. These homo- and heterodimeric proteins make extensive use of different DNA spacer lengths and sequences to directly affect the combinatorial assembly of the dimeric protein on DNA (11–13). Recent experiments on the PIT-1 homeodomain protein have also demonstrated an allosteric effect of DNA. A two-base pair change in the spacer not only changes the conformation of bound proteins but also the function of the proteins from a repressor to an activator (14).

Biochemical experiments have also hinted at a relationship between the DNA-bound conformation of NF-κB with its function. NF-κB binds with equal affinity to the κB sites from the Ig κ light chain gene enhancer (Ig-κB) and the interleukin-2Rα gene promoter (IL2Rα-κB) (15). However, only the Ig-κB site activates transcription potently in Jurkat phorbol ester-stimulated transfection experiments. Fujita et al. (16) used altered protease sensitivity patterns to demonstrate a unique p50 homodimer conformation when it strongly activates from Ig-κB that is absent when bound to H2-κB DNA from which it does not strongly activate.
"κDNA Is an Allosteric Regulator of Transcription"

The identity and arrangement of DNA binding sites for transcriptional activators in each complex enhancer appear to be gene-specific. For example, the HIV proximal promoter contains two identical κB sequences followed immediately by three binding sites for the transcription factor Sp1. The DNA binding domains of NF-κB and Sp1 were shown to cooperatively bind DNA (17). In this study, we sought to understand how HIV-κB DNA controls multiple activator assembly and transcription. We have endeavored to determine the roles of particular κB sites in effecting DNA conformation, interprotein interactions, and transcription from the HIV-LTR promoter. We also tested whether these κB sites are replaceable with other κB sites. To this end, we first determined the crystallographic structure of NF-κB p50/p65 heterodimer bound to the unique κB sequence from the human urokinase plasminogen activator gene promoter (uPA-κB) and compared it with the p50/p65 HIV-κB complex (2). Next, binding cooperativity was measured between NF-κB and itself when bound to two tandem HIV-κB sequences as well as between NF-κB and Sp1 when bound to two adjacent target sites from the HIV-LTR. Finally, we measured the transcriptional activation of hybrid HIV-LTR enhancers with the 3′ κB site converted to uPA-κB and to an interferon β gene enhancer κB (IFNβ-κB).

**EXPERIMENTAL PROCEDURES**

**Protein and DNA Purification**—The NF-κB heterodimer protein used in the uPA-κB complex crystallization contained co-expressed truncated murine p50 (residues 39–350) and p65 (residues 19–291). The heterodimer was purified according to previous published protocols (18). The peak fractions were concentrated, aliquoted, and stored at −80 °C, and used within 5 h of thawing.

The UPA-17 base pair DNA target site was synthesized as two single-stranded oligonucleotides via phosphoramidite synthesis with the following sequences: 5′-ctcggagaagtcga-3′ and 5′-tcgtacctctctcgag-3′ (Yale Howard Hughes Medical Institute Biopolymer/Keck Foundation Biotechnology Resource Laboratory). The DNA strands were purified and annealed as described earlier (2). The final double-stranded oligonucleotide was mixed with a 10% molar excess of the p50/p65 heterodimer. DNAs containing 5′-iodinated uracil bases were synthesized with the following sequences: UPA-2, 5′-p65 heterodimer and the DNA binding domain of p50/p65 and from the human urokinase plasminogen activator gene promoter (uPA-κB) and compared it with the p50/p65 HIV-κB complex (2). Next, binding cooperativity was measured between NF-κB and itself when bound to two tandem HIV-κB sequences as well as between NF-κB and Sp1 when bound to two adjacent target sites from the HIV-LTR. Finally, we measured the transcriptional activation of hybrid HIV-LTR enhancers with the 3′ κB site converted to uPA-κB and to an interferon β gene enhancer κB (IFNβ-κB).

**Crystallography and X-ray Diffraction Data Collection** — Crystals grew at 18 °C (0.08 × 0.08 × 0.3 mm) from 2-μl hanging drops in a final concentration of 6 mg/ml complex, 50 mM Na-MES (pH 6.5), 200 mM CaCl2, 0.05% β-octyl glucopyranoside, 0.05 mM sodium spermine, 10 mM dithiothreitol, and 10% polyethylene glycol 3350. Crystals with iodinated DNA grew in the same conditions. Before data collection, crystals were dialyzed at 4 °C over 2 days in 10 mM Tris–HCl, pH 7.5, 1 mM MgCl2, and 20% glycerol, mounted, and flash frozen in a liquid nitrogen stream. X-ray diffraction data were collected at 100 K using a MarResearch imaging plate system using x-rays from a large Rigaku rotation anode FR5 operated at 50 kV and 100 mA. The unit cell dimensions are as follows: a = 137.13, b = 179.70, c = 97.07 Å in the C2221 space group. There is one dimer in the asymmetric unit, with a solvent volume fraction of 68.5%. The data were indexed and integrated using DENZO (19) and scaled by SCALEPACK (20). The data collection and reduction statistics are summarized in Table I.

**Structure Solution and Refinement** — The UPA-4 crystal structure of the heterodimer-DNA complex was solved by molecular replacement method combined with multiple isomorphous replacement. Molecular replacement was performed with a p50/p65 heterodimer-DNA as the search model (2) in AMoRe (21) against the UPA-4 data set alone. The orientation and position of the molecular replacement solution was subsequently refined by rigid body refinement with CNS (22). We initially could not place the p50 N-terminal domain in the correct position. We performed multiple isomorphous replacement with two iodine derivatives (UPA-2 and UPA-5) to obtain additional phase information. Iodine sites were identified by difference Fourier synthesis, and then phasing parameters were refined and combined using MLPHARE (23). The figure of merit after phase combination was 0.59 for acentric and 0.74 for centric reflections. After histogram matching and solvent flattening, the electron density map allowed us to locate the N-terminal domain of p50.

Refinement was performed against the UPA-4 data set. Manual adjustments were made based on Fe-F, 2Fe-F, and difference maps using O (24). The parameter set of Engh and Huber was applied for the structure refinements. When individual isotropic temperature factors were included in the refinement, a final crystallographic R factor of 25.0% was achieved. The R free value of 29.1% was determined from PROCHECK (25) showed that 98% nonglycine residues were in most favored regions (77.5%) and additional allowed regions (20.3%) in both subunits. The final refinement statistics and stereochemical parameters are presented in Table I.

A lower resolution native data set was subsequently collected. Refinement was performed against this native UPA-17 data set in the same manner. The model was refined to a final R factor of 25.8% and an R free value of 30.3%. Ramachandran plots from PROCHECK (25) showed that 98% nonglycine residues were in most favored regions (72.0%) and additional allowed regions (26.0%) in both subunits. The final refinement statistics and stereochemical parameters are presented in Table I. No noticeable differences were observed in the refined native model compared with that of the refined derivative. Because the model obtained from the derivative data is higher in resolution with better statistics, we used this model for interpretation.

**Fluorescence Polarization** — To determine its HIV-κB DNA binding affinity, p50(39–350)/p65(19–291) heterodimer protein was serially diluted from 1 μM to 31 pm in FF buffer (50 mM NaCl and 10 mM Tris–HCl, pH 7.5) at 37 °C. The fluoroscence-labeled HIV-κB target DNA was added at 50 fmol total concentration with a Beacon 2000 Fluorescence Polarizer. After the addition of 0.1 mM fluorescein-labeled HIV-κB site DNA, the samples were incubated for 1 h at room temperature, and final readings were taken.

To determine the affinity of the protein for competitor DNA, p50(39–350)/p65(19–291) heterodimer protein was diluted to a final protein concentration of 40 nM and a final fluorescein-HIV-κB DNA concentration of 4 nm in 0.5 ml of FP buffer and blanked. After the addition of serially diluted unlabeled double-stranded competitor DNA with a maximum concentration of 25 μM (either UK19 (5′-TCTGAGGACCTTCTCTGATC-3′) or Δ20 (5′-TCTGAGGACCTTCTCTGATC-3′; κB sites underlined), the samples were incubated for 1 h at 25 °C, and final readings were taken. Fraction-bound DNA was plotted versus protein concentration and fitted to the following equation to determine the IC50 value: y = (x/IC50 + 1), where IC50 is the concentration (x) at which 50% of the labeled DNA is complexed with protein.

**Electrophoretic Mobility Shift Assay**—Single-stranded DNAs were synthesized off site (GenoSys) and annealed. They were then [32P]dATP-labeled with polynucleotide kinase (New England Biolabs) at 37 °C for 30 min and cleaned of free nucleotides (Qiagen). About 50 pmol of these DNAs were used per experiment (final concentration 1 μM). The HIV-κB p50/p65 heterodimer and the DNA binding domain of Sp1 (612–706 amino acids) were diluted in 5 mg/ml bovine serum albumin (Roche Molecular Biochemicals), 50 mM Tris–HCl (pH 7.5), and 1.0 mM zinc acetate. NF-κB was added in increasing total concentrations from 12.2 pm to 4.4 μM. Sp1, when present, was at a final concentration of 200 nM, enough Sp1 to saturate the DNA. After the DNA–protein complex was formed at 25 °C for 30 min in 1.0 mg/ml bovine serum albumin (Roche Molecular Biochemicals), 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1.0 mM zinc acetate, 0.1% Nonidet P-40, 5 mM β-mercaptoethanol, 10 mM glycerol, and 0.1 μg/ml poly(dI-dC) (Amersham Biosciences). Samples were loaded onto nondenaturing 6% acrylamide 0.5X TB gels that had been prerun at 4 °C with a constant 200 V. Gels were run at 4 °C with circulating 0.5× TB buffer for 3 h at a constant 200 V, dried on filter paper (Whatman), exposed to a PhosphorImager screen, scanned with a Molecular Dynamics 445 scanner, and quantified with the ImageQuant software. Equilibrium dissociation constants for NF-κB binding to DNA alone and Sp1-saturated DNA were calculated using a 1:1 binding model, A + B → AB, where A represents an NF-κB dimer and B represents DNA or Sp1-saturated DNA. EMSA experiments on κB binding were carried out in an identical manner, except for data analysis. This was a result of multiple migrating species for DNA bound by a single NF-κB dimer in the case of the hybrid DNAs. Gels were quantified via phosphorimaging (Molecular Dynamics 445 scanner) to produce background-subtracted values for each band. The ratio of ternary complex (DNA bound by two NF-κB dimers) versus binary complex (DNA bound by a single NF-κB dimer) dimerization value of 200 nM Sp1-saturated DNA at 25 °C for the wild type (HIV-HIV), IFNβ-HIV, and uPA-HIV targets. The binary bands for the uPA-HIV and IFNβ-HIV gels were taken as a sum of the two migrating species. All experiments were performed a minimum of three times.

**In Vitro Transcription Experiments**—The HIV-chloramphenicol acetyltransferase reporter construct was a gift from G. Nabel. Mutra-
heterodimer binds to a site-directed mutagenesis kit (Stratagene). The following sense strand primers and their corresponding antisense strands used for mutagenesis were synthesized off site (GenoSys) and had the following sequences: IFN-κB, 5'-gggaactctggaagactggagggtgc-3'; uPA-κB, 5'-gggaactctggaagactggagggtgc-3' (changes underlined). Mutations were confirmed via DNA sequencing.

Recombinant proteins were expressed and purified as described previously (26, 27). In vitro transcription reactions were carried out as follows. Template DNA (100 ng) was incubated with recombinant proteins for 30 min at room temperature followed by the addition of 40 μl of transcription mix containing 0.5 mg/ml bovine serum albumin, 5 μM dithiothreitol, 0.5 mM NTPs, 4 mM MgCl2, 35 mM HEPES, pH 8.2, 80 mM KCl, and 10 μl of HeLa nuclear extract (8 mg/ml). The reactions were incubated at 30°C for 1 h and stopped by the addition of 100 μl of mix containing 0.4 M sodium acetate, 10 mM EDTA, 0.2% SDS, 5 μg of tRNA, and 20 μg of proteinase K at 37°C for 15 min. The RNA was extracted by phenol/chloroform and ethanol-precipitated. The RNA pellet was resuspended in 20 μl of hybridization mix containing 40 mM PIPES, pH 6.4, 1 mM EDTA, 400 mM NaCl, 0.2% SDS, 0.1 pmol of γ-32P-labeled CAT primer followed by incubation at 37°C overnight. The hybridization reaction was precipitated by adding 180 μl of 0.5 M ammonium acetate and isopropyl alcohol. The DNA-primer hybrid was washed briefly in 50 mM sodium acetate, 10 mM EDTA, 0.2% SDS, 5 μg of tRNA, and 20 μg of proteinase K at 37°C for 15 min. The RNA was extracted by phenol/chloroform and ethanol-precipitated. The RNA pellet was resuspended in 20 μl of hybridization mix containing 40 mM PIPES, pH 6.4, 1 mM EDTA, 400 mM NaCl, 0.2% SDS, 0.1 pmol of γ-32P-labeled CAT primer followed by incubation at 37°C overnight. The hybridization reaction was precipitated by adding 180 μl of 0.5 M ammonium acetate and isopropyl alcohol. The DNA-primer hybrid was washed briefly in 50 mM sodium acetate, 10 mM EDTA, 0.2% SDS, 5 μg of tRNA, and 20 μg of proteinase K at 37°C for 15 min. The RNA was extracted by phenol/chloroform and ethanol-precipitated. The RNA pellet was resuspended in 20 μl of hybridization mix containing 40 mM PIPES, pH 6.4, 1 mM EDTA, 400 mM NaCl, 0.2% SDS, 0.1 pmol of γ-32P-labeled CAT primer followed by incubation at 37°C overnight. The hybridization reaction was precipitated by adding 180 μl of 0.5 M ammonium acetate and isopropyl alcohol. The DNA-primer hybrid was washed briefly in 50 mM sodium acetate, 10 mM EDTA, 0.2% SDS, 5 μg of tRNA, and 20 μg of proteinase K at 37°C for 15 min. The RNA was extracted by phenol/chloroform and ethanol-precipitated. The RNA pellet was resuspended in 20 μl of hybridization mix containing 40 mM PIPES, pH 6.4, 1 mM EDTA, 400 mM NaCl, 0.2% SDS, 0.1 pmol of γ-32P-labeled CAT primer followed by incubation at 37°C overnight. The hybridization reaction was precipitated by adding 180 μl of 0.5 M ammonium acetate and isopropyl alcohol. The DNA-primer hybrid was washed briefly in 50 mM sodium acetate, 10 mM EDTA, 0.2% SDS, 5 μg of tRNA, and 20 μg of proteinase K at 37°C for 15 min.

RESULTS

NF-κB Adapts Variable Conformations on Different κB Sites—We endeavored to determine whether the NF-κB p50/p65 heterodimer binds to a κB site other than the HIV-κB with a different conformation. The target site selected for this study was from the urokinase plasminogen activator (uPA) promoter (28). This site was selected because a comparison of this target site with the HIV-κB site shows four base pair differences at the −1, 0, +1, and +3 positions (Fig. 1). Two of these differences are in positions central to mediating base-specific contacts in the HIV-κB structure (2) and were expected to produce the most observable changes in conformation. Both the structural and the biochemical experiments were performed with the p50/p65 RHR that is sufficient for DNA binding. The structure of the p50/p65 RHR bound to a DNA target 17 base pairs long containing the uPA-κB site was solved to 2.7-A resolution using molecular replacement and multiple isomorphous replacement techniques (Table I, Fig. 2A). The heterodimer bound to the uPA-κB site assumes a different and unique global conformation compared with the HIV-κB-bound structure (2) (Fig. 2B). Superposition of the dimerization domains of the two structures reveals, relative to the Ig-κB bound structure, a rotation of the uPA-κB-bound p65 NTD by 10.3° and a translation by 8.8 Å, while the p50 NTD is rotated by 6.5° and translated by 7.1 Å.² In both uPA-κB- and Ig/HIV-κB-bound complexes, the same p50 residues directly contact the 5'-DNA half-site bases, His-64, Arg-56, Arg-54, Glu-60, Lys-241, and Tyr-57. However, the detailed chemistry of these interactions is different (Fig. 2C). Arg-54 makes bidentate contacts with the N-7 and O-6 atoms of G-4 instead of those of G-3 as previously observed. Arg-56 no longer makes bidentate contacts but now only contacts the N-7 atom of G-4. Glu-60 also shifts by contacting the N-4 atom of C-3 instead of both the N-4 atoms of C-3 and the backbone of Tyr-57 as observed in the HIV-κB structure. Lys-241 now contacts both O-4 atoms of T-1 and T-2 instead of just G-1. The p65 subunit binds to the 3' DNA half-site via Arg-35, Glu-39, and Arg-187 and makes stacking contacts through Tyr-36 (Fig. 2D). noteworthy are the changes in contacts at the +3 base pair, which has been mutated from a G:C base pair in the HIV-κB to a T:A base pair in the uPA-κB. The side chain of Arg-35 bends back into the protein, not making hydrogen contacts to the DNA and making van der Waals contacts. Glu-39 also reorients, contacting only the N-6 of A-3 and Arg-35 and losing a contact to the backbone of Tyr-36. Arg-187 and Tyr-36 maintain similar contacts to the DNA in both structures. DNA backbone contacts are also not conserved between the structures (Fig. 2E). There are only 12 backbone contacts made in

![Comparison of κB DNA sites.](https://www.jbc.org/)  
FIG. 1. Comparison of κB DNA sites. Shown are κB sites from the immunoglobulin κ light chain enhancer gene/HIV promoter (HIV), the urokinase plasminogen activating gene (uPA) promoter (28). This site was selected because a comparison of this target site with the HIV-κB site shows four base pair differences at the −1, 0, +1, and +3 positions.

| Crystal | Space group | Resolution | Reflections (total/unique) | Rfre | Completeness | Rsym b |
|---------|-------------|------------|---------------------------|------|--------------|-------|
| A       |             |            |                           |      |              |       |
| UPA-17  | C222        | 2.8        | 258,270/27,886            | 19.1 | 98 (91)      | 10.3 (57.3) |
| UPA-2   | C222        | 3.1        | 169,314/20,814            | 8.5  | 95 (76)      | 9.2 (46.0)  |
| UPA-4   | C222        | 2.7        | 254,045/35,470            | 13.8 | 98 (89)      | 6.4 (31.5)  |
| UPA-5   | C222        | 2.7        | 215,647/30,951            | 15.9 | 96 (97)      | 7.6 (36.6)  |

| Refinement | Resolution range | Reflections | No. of atoms | r.m.s. deviations | Bond length | Bond angle | Rcrystcd | Rfreee |
|------------|------------------|-------------|--------------|------------------|-------------|-----------|----------|--------|
| A          |                  |             |              |                  |             |           |          |        |
| UPA-17     | 30.0–2.8         | 25,583      | 5406         | 0.0074           | 1.49        | 25.8      | 30.3     |        |
| UPA-4b     | 20.0–2.7         | 29,985      | 5315         | 0.0072           | 1.33        | 25.0      | 28.4     |        |

a Parentheses denote highest resolution shell.  
b Rsym = |Iobs − Iexp| / |Iexp|.  
c See “Experimental Procedures,” Protein and DNA Purification for DNA sequence and derivative information.  
d Rcryst = |Fo − Fc| / |Fo|.  
e Rfree was calculated with 5% of data.
recent x-ray structure solution of NF-κB have shown that NF-κB
binds to different protein conformations on different DNAs. Both x-ray
observations from crystallographic structures, which show dif-
ferent mobilities (Fig. 4C). This result confirms that the re-
nounced number of direct contacts in the NF-κB-uPA-κB complex
does not necessarily lower binding affinity.

Variations in κB Sequence Marginally Affect Interactions
between NF-κB and Sp1—Having crystallographically deter-
mined distinct conformations and biochemically observed similar
binding affinities, we tested if this variability in NF-κB structure
affects multiprotein assembly on and subsequent transcription from the HIV LTR proximal promoter. The HIV LTR proximal promoter contains two NF-κB binding sites ad-

NF-κB Binds to HIV-κB, uPA-κB, and IFNβ-κB and with
Similar Affinities—To test if the direct DNA contacts made by
the protein in the uPA-κB complex affect the affinity of the
NF-κB-uPA-κB complex, we measured the relative binding affinities by using fluorescence polarization competition assays to determine IC₅₀ values, the concentration of competitor DNA at which 50% of the NF-κB-fluorescein-labeled DNA reference complexes were displaced. The IC₅₀ values were found to be similar for uPA-κB (28 nm), HIV-κB (20 nm) (Fig. 3), and IFNβ-κB, a κB site present in the enhancer of the interferon β gene (data not shown). We have also measured the apparent equilib-
rium dissociation constant of NF-κB for these κB sites (Kᵦ) through EMSAs (Fig. 4A). These experiments show that direct binding of NF-κB to the three different DNAs also occurred with similar affinity (Fig. 4B). The apparent Kᵦ values for the HIV-κB site, the uPA-κB site, and the IFNβ-κB site are 0.8, 1.2, and 0.8 nM, respectively. Additional gel shift experiments of NF-κB binding to the three κB sites show that the complexes run with different mobilities (Fig. 4C). This result confirms observations from crystallographic structures, which show different protein conformations on different DNAs. Both x-ray crystallographic experiments and biochemical DNA bending experiments (29) have shown that NF-κB heterodimer binds different κB DNAs with varying degrees of bend angle. The recent x-ray structure solution of NF-κB bound to the IFNβ-κB site demonstrates that the protein can assume yet another distinct conformation. Similar affinities suggest that the re-
3 B. Berkowitz et al., submitted for publication.
It has been shown that the HIV proximal promoter is sufficient to drive TATA box-dependent transcription. We tested if the conversion of an HIV-\(\kappa\)B/H9260B into a uPA-\(\kappa\)B/H9260B or an IFN\(\beta\)-\(\kappa\)B site would alter the cooperative interactions between NF-\(\kappa\)B p50-p65 and Sp1 and between two molecules of NF-\(\kappa\)B bound to the tandem \(\kappa\)B sites.

To test the cooperativity between NF-\(\kappa\)B and Sp1 in the wild type DNA, we employed a test probe containing the 3\textquotesingle \(\kappa\)B site adjacent to three Sp1 binding sites (Fig. 4A). It has been shown that the HIV proximal promoter is sufficient to drive TATA box-dependent transcription. We tested if the conversion of an HIV-\(\kappa\)B into a uPA-\(\kappa\)B or an IFN\(\beta\)-\(\kappa\)B site would alter the cooperative interactions between NF-\(\kappa\)B p50-p65 and Sp1 and between two molecules of NF-\(\kappa\)B bound to the tandem \(\kappa\)B sites.

To test the cooperativity between NF-\(\kappa\)B and Sp1 in the wild type DNA, we employed a test probe containing the 3\textquotesingle \(\kappa\)B site...
and the nearby 5’ Sp1 site and made changes to the 3’ κB site so that it became a uPA-κB or and IFNβ-κB site. In this experiment, increasing amounts of NF-κB were added to Sp1-saturated DNA. We observed that in the presence of Sp1, NF-κB’s affinity for the HIV-κB DNA improves by only 1.5-fold ($K_D = 0.6 \, \text{nm})$. NF-κB’s affinity is only improved 1.2-fold for uPA-κB ($K_D = 1.0 \, \text{nm}$) and 1.4-fold for the IFNβ-κB site ($K_D = 0.7 \, \text{nm}$) in the presence of Sp1. The results of these experiments suggest that assembly between NF-κB and Sp1 is only marginally affected when the HIV-κB is converted to either a uPA-κB or an IFNβ-κB site (Fig. 4D).

**Variations in κB Sequence Affect Conformation and Assembly of NF-κB on Tandem Sites**—To observe the interprotein interactions of NF-κB with itself, we used DNAs containing two tandem κB sites in EMSA experiments. On the native DNA, the second NF-κB molecule binds to the binary NF-κB-DNA complex in a defective manner with no apparent cooperativity (Fig. 4E). Even at a concentration of 4 μM, ternary complex formation did not reach saturation. Because we could not satisfactorily calculate the anticooperativity, and in order to show the differences in binding between the HIV-κB and hybrid DNAs, we compared the ratio of ternary to binary complex formed on each DNA (Fig. 4F). Binding of a second NF-κB molecule to a DNA with one of the κB sites swapped to a uPA- or IFNβ-κB site occurs with significantly lower affinity than to the native DNA. At all tested protein concentrations, the observed ratio of ternary to binary complex exhibits higher levels of complex formation in the native context (Fig. 4F). These results clearly demonstrate that small changes in nucleotide sequence can have profound effects on cooperativity. Because these changes are localized to the κB site, we suggest that differential cooperativity of binding is due to conformational changes in NF-κB p50-p65 heterodimer binding. These EMSA experiments have also been performed with full-length recombinant p50-p65 heterodimer and demonstrate no differences in the DNA interactions (data not shown).

In the gels in which the double κB DNA has a 3’ κB site swapped with a uPA- or IFNβ-κB site, the binary complex runs as a doublet (Fig. 4D). Each band in the doublet represents one NF-κB dimer bound to one of the two κB sites. Because the protein binds to and bends each κB site within the hybrid DNAs with different conformations and to different degrees, the two protein-DNA complexes migrate differently (Fig. 4E). The ternary complex, in which both κB sites are occupied on the DNA, migrates as a single species. This experiment confirms our observations from the prior EMSA experiments.

**Alterations in κB Sequence Affect Transcriptional Activation from the HIV-LTR Promoter**—To test if the differential binding profiles of NF-κB p50-p65 and Sp1 on these native and altered HIV-LTRs also affect its transcription, we performed in vitro experiments with the HIV-LTR CAT system (17). The plasmid contains a CAT reporter gene driven by the complete proximal κB site. In vitro transcription levels were measured for all three constructs with increasing NF-κB concentrations and compared with that of a control lacking NF-κB. At lower protein concentrations, we observed that the native template is the most efficient, whereas the uPA and IFNβ templates are 3–4-fold defective (Fig. 5).

**DISCUSSION**

The coordinated interplay of multiple DNA-bound activators, coactivators, and basal transcription components facilitates the
various observed and distinct patterns of transcriptional activation. Assembly of the multiprotein enhanceosome is influenced by the DNA flexibility. Rel dimers have been previously shown to assume variable conformations upon binding to different but related NF-κB sites both biochemically (16) and structurally (2). In this study, we have determined the three-dimensional structure of NF-κB p50-p65 bound to the uPA-κB site and observe that the overall conformation of this complex is different than that of our previously determined Ig/HIV-κB crystal complex. This paper provides the first crystallographic evidence of variable conformations in the Rel family of transcription factors, that they cause allosteric effects in multiprotein assembly, and that they are functionally linked to altered transcriptional activities.

A comparison of the Ig/HIV-κB and uPA-κB sequences shows differences at four base pairs of which two are not involved in direct hydrogen bonding contacts with the protein. The two differing base pairs between the Ig/HIV-κB and IFNβ-κB sites also do not participate in hydrogen bonds with the protein. While sequence alterations in the protein-interacting bases have effects on the protein side chain conformations, non-protein-interacting bases may also influence binding in another manner. They may instead change the sequence-dependent structure of DNA and thus the modes by which NF-κB, facilitated by its modular architecture, recognizes DNA (8–10). In the same manner, alterations in the DNA sequence flanking the recognition site could also induce conformational changes in NF-κB-DNA complexes. In our two crystallographic models, these cumulative effects in the DNA targets produce conformational changes in the proteins. As a consequence of conformational change, cooperativity of interactions between NF-κB and nearby bound proteins is altered. Whereas smaller effects are observed in the interactions with Sp1, significant effects are seen in interactions with a neighboring NF-κB molecule. An alteration of conformation influenced by the DNA sequence is also evident in the gel shift experiments. When either one of the two identical κB sites in the native HIV LTR is bound by a single heterodimer, the shifts are identical. In contrast, two different shifts are observed when one of the sites is mutated in the hybrid HIV LTRs. Differential migration of these complexes indicates that the conformation of NF-κB-HIV-κB, NF-κB-IFNβ-κB, and NF-κB-uPA-κB complexes are truly distinct.

Why does the cooperativity between two NF-κB molecules alter for hybrid κB DNA? There are two possible causes for the lack of observed cooperativity: indirect readout of the DNA or steric clash between the proteins. The structures of unbound NF-κB sites are different from standard B-DNA as evidenced by NMR and CD experiments (8–10, 30). Presumably, the dynamics of κB sites vary from one another, which explains why the conformations of various κB sites are different when bound to the same NF-κB dimers. This structural property of κB sites may explain how binding of a NF-κB molecule to one NF-κB site on the DNA could induce a structural change in a second heterologous NF-κB site differently than in a wild type NF-κB site.

The possibility of steric clash between the two proteins is also a possibility. A straightforward model was generated of two NF-κB heterodimers from the Ig/HIV-κB-DNA bound crystal structure (Protein Data Bank accession code 1VKX) and spaced correctly on a 26-base pair B-DNA containing the HIV LTR sequence. This model of the protein:HIV LTR DNA complex shows the phasing of the two heterodimers, reveals a close proximity between the L1 loops, and therefore implicates them as protein regions that interact with the neighboring molecule (Fig. 6). Variable conformations in the two NF-κB molecules through this loop might alter favorable interactions when complexed to the native tandem κB sites to unfavorable ones as in hybrid tandem κB sites. Movements in the L1 loop may also prevent ideal interactions to the DNA.

We find that these DNA-induced conformational changes affect NF-κB’s transcriptional activation from promoters containing these sites. The use of HIV-chloramphenicol acetyltransferase reporter constructs and derived hybrids permits in vitro experimentation in the context of an intact HIV-LTR promoter. We observe that the hybrid promoters are incapable of maintaining the high levels of transcription observed in the native promoter although NF-κB binds to these altered κB sites with near equal affinity. This effect is at least partly due to defects in enhanceosome assembly. Whereas the DNA binding domains of p65 and Sp1 were shown to be sufficient for interaction on the HIV LTR (17), it remains to be defined what contribution the activation domain of p65 makes in these interactions. The p65 activation domain is also known to specifically interact with other basal and coactivator proteins such as CREBP-binding protein, human TBP-associated factor II31, and human TBP-associated factor II80 (31, 32).

The greatest difference in the in vitro transcription levels observed between the native and the hybrid HIV LTR promoters is at the lower concentration range of 0.2–2 μg/ml. This lower range is more physiologically relevant than one might initially believe, since the effective concentration of p50-p65 heterodimer available for any given κB site is much less than 4–5 μg/ml, the observed cellular concentration based on the observations by Hottiger et al. (33) that roughly 120,000–125,000 p65 molecules are present per cell. NF-κB regulates several hundred κB sites in the mammalian genome, and Rel subunits are present in a number of different dimer combinations. Even the small differences in observed transcriptional activation at the lower NF-κB concentrations are significant, since sensor chip experiments may show only a 2.5–3-fold difference between noninduced and induced cell states (34).

The specific DNA-bound conformation that a regulator protein adopts is critical in forming a specific enhanceosome with distinct effects on transcriptional activity. This report demonstrates an allosteric mechanism in NF-κB mediated by conformational changes. Consistent with this allosteric role of DNA, we observe that the κB site of an enhancer cannot be interchanged with other κB sites to produce the same biological effect when activated by the NF-κB p50-p65 heterodimer. Considering that a plethora of genes are regulated specifically by NF-κB p50-p65 heterodimer-containing enhanceosomes, a few of which include IFNβ (35, 36), IRF-1 (37), GM-CSF (38), and the interleukin-8 (39), the effect of allosterically regulating κB DNAs on the cells can be profound. Transcriptional activation, however, remains far from exhaustively understood and a region in which we can still expect more new insights.

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