

SURVEY AND SUMMARY

Genome reading by the NF- κ B transcription factors

Maria Carmen Mulero^{1,†}, Vivien Ya-Fan Wang^{2,†}, Tom Huxford³ and Gourisankar Ghosh^{1,*}

¹Department of Chemistry and Biochemistry, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093, USA, ²Faculty of Health Sciences, University of Macau, Avenida da Universidade, Taipa, Macau SAR, China and ³Structural Biochemistry Laboratory, Department of Chemistry and Biochemistry, San Diego State University, 5500 Campanile Drive, San Diego, CA 92182, USA

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ABSTRACT

The NF- κ B family of dimeric transcription factors regulates transcription by selectively binding to DNA response elements present within promoters or enhancers of target genes. The DNA response elements, collectively known as κ B sites or κ B DNA, share the consensus 5'-GGGRNNYCC-3' (where R, Y and N are purine, pyrimidine and any nucleotide base, respectively). In addition, several DNA sequences that deviate significantly from the consensus have been shown to accommodate binding by NF- κ B dimers. X-ray crystal structures of NF- κ B in complex with diverse κ B DNA have helped elucidate the chemical principles that underlie target selection *in vitro*. However, NF- κ B dimers encounter additional impediments to selective DNA binding *in vivo*. Work carried out during the past decades has identified some of the barriers to sequence selective DNA target binding within the context of chromatin and suggests possible mechanisms by which NF- κ B might overcome these obstacles. In this review, we first highlight structural features of NF- κ B:DNA complexes and how distinctive features of NF- κ B proteins and DNA sequences contribute to specific complex formation. We then discuss how native NF- κ B dimers identify DNA binding targets in the nucleus with support from additional factors and how post-translational modifications enable NF- κ B to selectively bind κ B sites *in vivo*.

INTRODUCTION

Nuclear factor κ B (NF- κ B) is a family of dimeric DNA binding transcription factors that modulate diverse biolog-

ical responses to cellular stress by regulating the expression of hundreds of effector genes controlling these processes (1). Under the typical conditions of a resting cell, most NF- κ B is rendered inactive through its noncovalent association with a class of inhibitor proteins known collectively as I κ B. Even under these steady state conditions, however, a small pool of NF- κ B remains free of I κ B and directs expression of genes that help maintain cellular homeostasis (2,3). Although NF- κ B:I κ B complexes localize predominantly to the cytoplasm of resting cells, a small fraction can be detected within the nucleus where, by virtue of their inability to bind DNA, the I κ B-bound NF- κ B are mostly inactive. There are exceptions to this rule since certain NF- κ B:I κ B complexes do, in fact, exhibit DNA binding activity once the complex-associated I κ B is modified post-translationally in response to a specific stimulus (discussed later). The pool of free, active nuclear NF- κ B dimers becomes amplified by several-fold, however, upon cell stimulation by a vast number of diverse chemical, biological, or environmental factors.

Five related polypeptide subunits constitute the mammalian NF- κ B family: p50/NF- κ B1, p52/NF- κ B2, p65/RelA, c-Rel and RelB (Figure 1). Each shares high amino acid sequence conservation throughout a large portion of roughly 300 residues in length located near their N-termini and referred to as the Rel Homology Region (RHR). This RHR is responsible for sequence-specific DNA binding, protein dimerization and I κ B binding. These proteins can be further divided into two sub-classes: the NF- κ B p50 and p52 subunits belong to class I by virtue of their not possessing a transcriptional activation domain (TAD). The other three family members, RelA, c-Rel and RelB (class II), each contain a TAD within their respective C-terminal portions. The NF- κ B p50 and p52 subunits are generated by partial proteolytic processing of the inhibitory p105 and p100 proteins, respectively. The

*To whom correspondence should be addressed. Tel: +1 858 822 0469; Email: gghosh@ucsd.edu

†The authors wish it to be known that, in their opinion, the first two authors should be regarded as Joint First Authors.

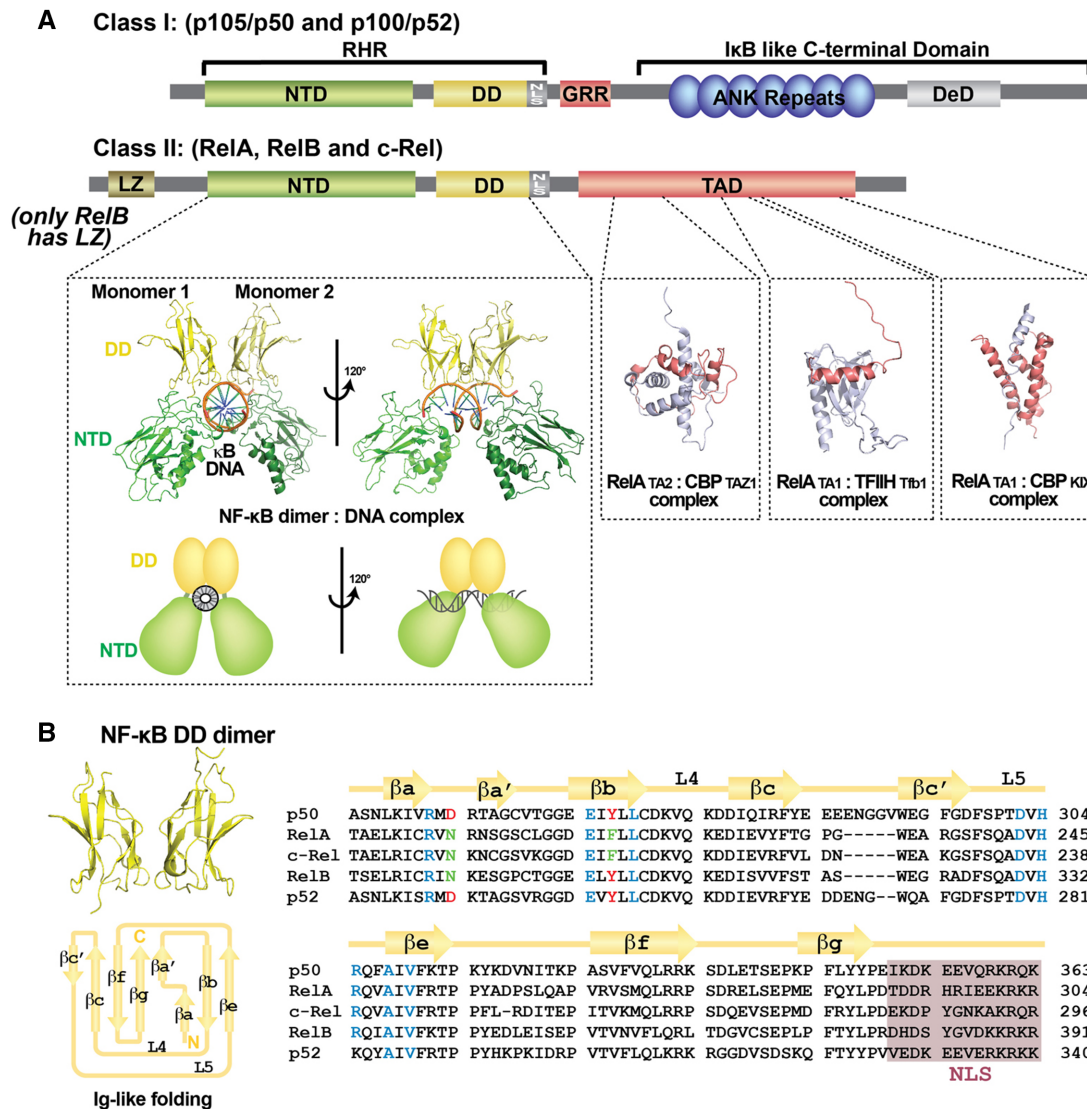


Figure 1. Domain organization of the NF- κ B family. (A) The upper panel contains domain schematic diagrams for NF- κ B family members. The p50 and p52 subunits (Class I) are generated from larger precursors (p105 and p100, respectively). The precursors, which contain an Ankyrin repeat domain (ARD) in their C-termini, function as inhibitors of NF- κ B. The precursors also contain a glycine rich region (GRR) which connects the RHR and ARD and a death domain (DeD). The Rel homology region (RHR) is folded into two distinct domains, N-terminal domain (NTD) and dimerization domain (DD). All NF- κ B proteins contain a nuclear localization signal (NLS). NF- κ B RelA, RelB and c-Rel subunit proteins (Class II) contain a transactivation domain (TAD). RelB has an N-terminal leucine zipper (LZ) domain. Depicted within the lower left panel is a ribbon diagram representation of the X-ray crystal structure of NF- κ B p50 homodimer bound to a κ B DNA in two different orientations (PDB ID: 1NFK). The complex displays its characteristic 'butterfly-like' shape with the DNA in the center and the two NF- κ B monomers embracing it. The three panels to the right depict solution structures of RelA TAD (TA1 and TA2 in red) bound to different proteins (TFIIH and CBP in grey) (PDB ID: 2LWW, 5URN and 5U4K). (B) Ribbon diagram (above left) and topology map (below left) of the NF- κ B DD immunoglobulin (Ig)-like fold (PDB ID: 1MY5). Right, sequence alignment of the DD of the NF- κ B subunits. Secondary structures and connecting loops are drawn above the sequences. The NLS is highlighted in purple. Residues critical for dimer formation are colored (invariant residues are blue and conserved residues are red or green).

p50 and p52 subunits terminate with a short ~ 70 residues long segment rich in glycine residues.

Since its original discovery in 1986 as a nuclear factor with binding specificity for a DNA element within an intronic enhancer of the kappa light chain gene in B cells (4), NF- κ B has served as a treasure trove for the discovery of novel biochemical mechanisms in intracellular signal transduction. As examples, the study of NF- κ B regulation has contributed directly to our current understanding of effector modulation and subcellular localization via protein-

protein interaction, protein kinase activation and substrate specificity, phosphorylation-dependent ubiquitylation, partial and complete processing of signaling molecules via proteasomes, and the use of alternative ubiquitin linkages as components of innate immune signaling. Many excellent reviews have been written that highlight the molecular mechanisms of these critical processes (5-9). However, fundamental questions remain surrounding the mechanisms by which active NF- κ B functions within the nucleus to elevate the expression of select target genes. In this review, we focus on

mechanistic details of DNA binding by NF- κ B dimers with emphasis on correlating current genome-wide and cellular observations to *in vitro* biochemical as well as structural and biophysical studies.

NF- κ B PROTEIN STRUCTURE

The 3D structures for the RHR of all five mammalian NF- κ B subunits have each been determined by X-ray crystallography (10–16). As predicted from their high degree of primary amino acid sequence similarity, their folded structures are also highly conserved (Figure 1A). The RHR contains two separately folded domains: an N-terminal domain (NTD), which is primarily responsible for sequence-specific DNA binding, and a C-terminal dimerization domain (DD). A short, ~10 amino acids long linker connects the two folded domains. A final conserved stretch of ~20 amino acids C-terminal to the DD, which contains the type I nuclear localization signal (NLS), is disordered in free or DNA-bound NF- κ B but converts to an alpha-helical secondary structure upon I κ B binding (17–19).

Each DD adopts a rendition of the common immunoglobulin (Ig)-like fold in which one four-stranded anti-parallel β -sheet packs against another three-stranded anti-parallel β -sheet (Figure 1B) (20). Unlike the Ig domains of antibodies, no cysteinyl disulfide bonds stabilize the folded DD. NF- κ B dimerization results from the juxtaposition of two Ig-like dimerization domains with C₂ symmetry (20,21). Although assembly of fifteen distinct NF- κ B homo- and heterodimers is possible through the pairwise combination of five NF- κ B subunits, not every potential dimer is observed *in vivo* (Figure 2). Sequence variations at the dimer interface support a mechanism for preferential homo- and heterodimer formation through amino acid side chain complementarity (Figure 1B) (22–24). But residues outside of the subunit interface also play important roles in modulating dimer stability and dictating preferred dimer combinations (21,23,25,26). The precise mechanism by which these distal residues function through space to control NF- κ B subunit dimerization remains unclear. In addition to the direct and indirect effects of specific amino acids within the DD, regulated synthesis/degradation of individual subunits and preferential binding by I κ B inhibitors also serve to bias the assembly of particular NF- κ B dimers within the context of the cell (27).

Of the NF- κ B dimers with potential to activate gene transcription, the p50:RelA heterodimer is ubiquitous and is involved in most biological activities associated with NF- κ B (28,29) (Figure 2). The RelA:RelA homodimer is less abundant, but also plays critical roles. Indeed, RelA:RelA can compensate for the loss of p50:RelA heterodimer in p50 null mice. Consequently, despite their increased susceptibility to infection, p50 subunit knockout mice survive into adulthood (30). Like RelA, c-Rel preferentially forms two dimers: p50:c-Rel heterodimer and the c-Rel:c-Rel homodimer (31). c-Rel protein expression has been analyzed via the Human Protein Atlas Database and was shown to be expressed in hematopoietic cells as well as in cardiac tissue, hepatocytes and keratinocytes (www.proteinatlas.org/ENSG00000162924-REL/tissue) (32). RelB also preferentially forms two dimers, though both p50:RelB and

p52:RelB are heterodimers (16,23,33). Whereas p50:RelA is the principal end product of induced NF- κ B transcriptional activity via canonical signaling, the p52:RelB heterodimer becomes active as a result of signaling through the ‘non-canonical’ (also known as ‘alternative’) NF- κ B pathway (34–38). Finally, though they lack C-terminal TADs and, consequently, inherent transcriptional activation potential, both p50:p50 and p52:p52 homodimers can activate or inhibit expression of select target genes through their association with I κ B proteins Bcl-3, I κ B ζ and I κ BNS. Gene knockout studies in mice indicate complicated, context dependent *in vivo* roles for these I κ B proteins in modulating NF- κ B-dependent gene expression (39–41). Though structurally similar to prototypical I κ B inhibitors, Bcl-3, I κ B ζ and I κ BNS interact only weakly with RelA, c-Rel or RelB (42–44). On account of their propensity to accumulate in the nucleus, Bcl-3, I κ B ζ and I κ BNS are referred to collectively as ‘nuclear I κ B’.

A STRUCTURAL PERSPECTIVE ON DNA RECOGNITION BY NF- κ B

κ B DNA *in vitro* and *in vivo*

NF- κ B dimers bind with sequence specificity to double-stranded DNA elements that vary in length from between 5 and 11 base pairs (bp). Early comparisons of the first DNA sequences demonstrated that NF- κ B binds to the consensus sequence: 5'-GGGRNWYYCC-3', where R = A or G; N = A, C, G, or T; W = A or T; and Y = C or T (Figure 3) (45). The subsequent identification of new NF- κ B DNA binding sites has broadened the consensus to 5'-GGGNNNNCC-3' (46). DNA from within gene enhancer regions that meet this later consensus and that can be shown experimentally to drive NF- κ B-dependent reporter gene expression are termed ‘ κ B DNA’ or ‘ κ B sites.’

The critical feature of the consensus κ B DNA sequence is the presence of a series of G and C nucleotide bases at the 5' and 3' ends, respectively, while the bases at the central portion display greater variation. Assuming the flanking G/C regions remain constant, the observed sequence variation within the central portion leads to three broad classifications of sequences: (i) sequences rich in AT, wherein all five central bp are A or T, (ii) sequences rich in GC and (iii) sequences with mixed AT and GC. Hundreds of such sequences belonging to all three classes have been identified by sequencing and confirmed experimentally, and the total unconfirmed κ B sites detected by computational methods alone number in the thousands (47).

Many of these hypothetical κ B sites exhibit significantly greater sequence variation than allowed by the original consensus κ B DNA. Of particular interest among these predicted sequences are a class of κ B DNA that display only partial (~5 bp) consensus such as those present in the promoters of CCND1 (GGGGACTTTT) and CCR7 (GGGGCTTTT) genes (48). Taking these κ B DNA into consideration, an alternative κ B DNA ‘half site’ consensus is 5'-GGGRNNYNNN-3' (Figure 3).

The critical issue to consider is whether the NF- κ B DNA response elements *in vivo* follow the same general features of κ B sites characterized *in vitro*. The widely used genome-wide method for motif identification is chromatin

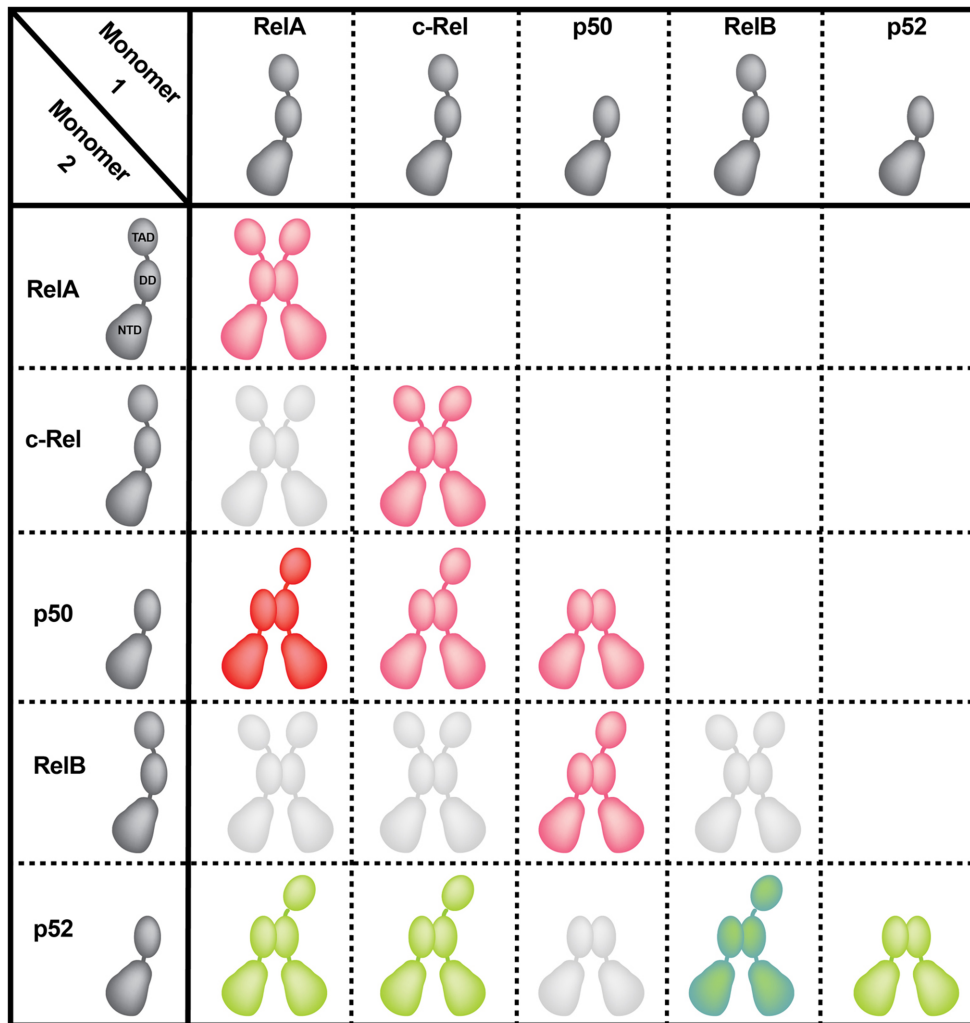


Figure 2. NF- κ B dimers. Cartoon representations of all possible NF- κ B homo- and heterodimer combinations. Dimeric NF- κ B proteins involved in canonical signaling are colored red with the ubiquitous p50:RelA heterodimer bolded for emphasis (29). Dimers that function in response to non-canonical (alternative) NF- κ B signaling are colored in green with p52:RelB, the predominant NF- κ B dimer of this pathway, darkened for emphasis (38). NF- κ B dimers not observed in cells are colored in light grey (26).

immunoprecipitation coupled to high throughput sequencing (ChIP-Seq). Over the past 15 years, ChIP-Seq studies, notably by Encyclopedia of DNA Elements (ENCODE) Project Consortium, have identified DNA motifs bound by nearly all transcription factors including NF- κ B (49–51). Although this method is technically limited in its ability to determine DNA binding motifs with absolute precision (52), it has generated a wealth of valuable data. As expected, the NF- κ B dimers associate with consensus κ B sites in addition to binding at sites that display only half-site consensus (53–56). Some of these κ B sites (GGGGATTTT or GGGGGTTTT) appear to be strong binders on the basis of the z-score of ChIP peaks (56), but they are weaker binding sites for NF- κ B p50:RelA heterodimer *in vitro*. Conversely, several consensus sites are only weakly recognized by NF- κ B *in vivo*. The mechanism by which NF- κ B binds with either high or low affinity to ‘partial’ κ B DNA sequences vs. consensus κ B DNA, respectively, *in vivo* remains unclear. Intriguingly, however, NF- κ B also binds to sites that pos-

sess no κ B consensus (53–55). Therefore, *in vitro* data do not fully capture the complexity of DNA recognition by the NF- κ B dimers *in vivo*. We discuss these aspects of NF- κ B-mediated gene regulation *in vivo* later in this review.

NF- κ B:DNA complexes

Successful determination of 3D structures of several NF- κ B RHR dimers in complex with diverse κ B DNA by X-ray crystallography has helped establish the chemical principles for sequence specific DNA recognition by NF- κ B (10,11,13–16,33,57–62). In general, κ B DNA is pseudo-symmetric and the RHR of each NF- κ B monomer binds to one κ B DNA half site. In its DNA-bound conformation, the NF- κ B subunit NTD, which also adopts a derivative Ig-like fold, positions itself such that an extensive basic (positively charged) surface counteracts the acidic phosphate-ribose DNA backbone. Movement of the NTD relative to the DD is afforded by virtue of the short stretch of 10 or so amino acids that link the two domains. This potential for

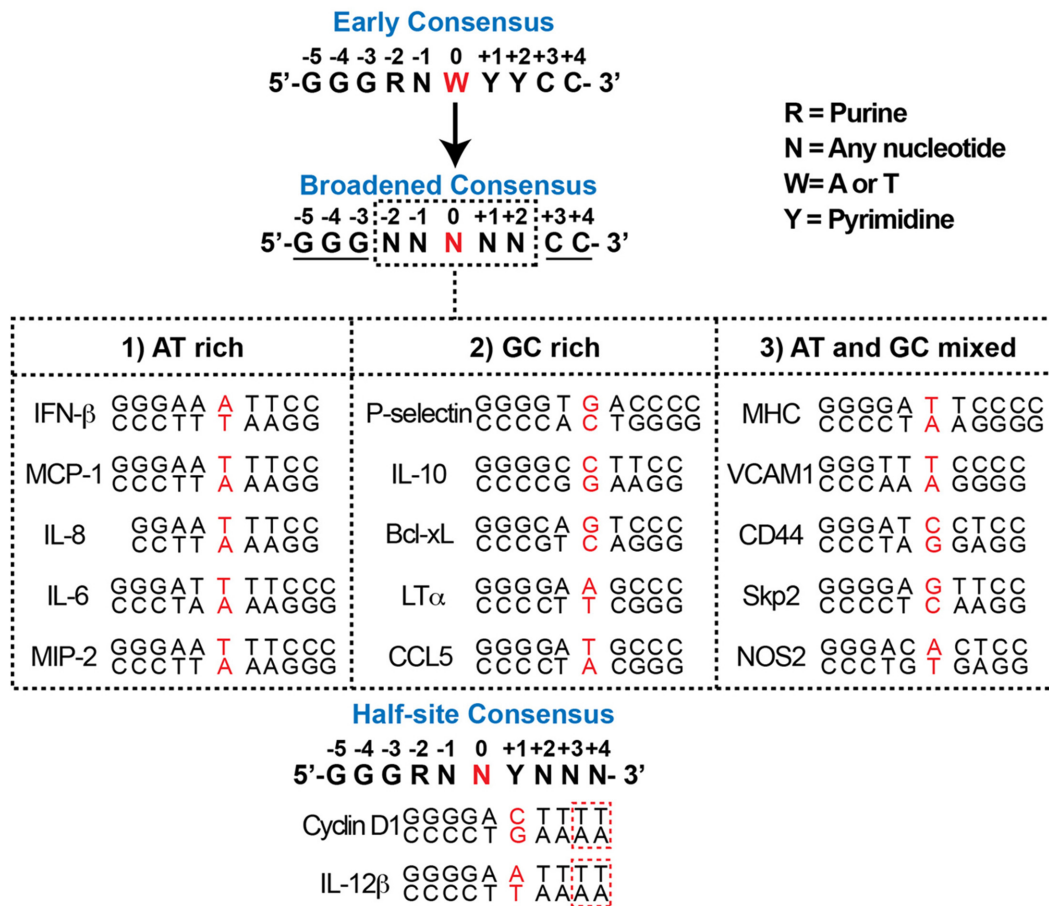


Figure 3. Classification of different types of κB DNA sequences. Top panel, the κB DNA sequence, as defined during the late 1980s, is labeled as ‘Early Consensus.’ This consensus was ‘Broadened’ in the late 1990s after the discovery of NF-κB binding to novel sequences. The central base pair is highlighted in red. Conserved flanking residues are underlined. Variable central portion residues are boxed in black. Middle panel, κB DNA sequences can be sorted into three classes with respect to the nature of the nucleotide sequences within the central portion. The central base pair is highlighted in red. Bottom panel, two examples of NF-κB target genes display that only half consensus κB DNA (48). Nucleotides that do not match with the κB DNA consensus sequence are boxed in red.

independent domain motion allows for the RelA NTD to move ~40 Å and to rotate by nearly 180° upon binding the IκBα inhibitor protein, as evidenced by NF-κB:IκBα complex X-ray crystal structures (17,18).

Amino acid side chains from the immunoglobulin (Ig)-like NTD and the interdomain linker (loop L3) of each NF-κB RHR mediate all of the direct contacts to specific DNA bases (Figure 4). The NF-κB dimer interface remains unchanged upon κB DNA binding, and several additional DNA backbone interactions involve residues from both the NTD and DD. The mode of DNA binding by the NF-κB RHR was unique at the time of its determination because all of the DNA contacts were mediated by amino acids on loops that connect β-strand elements of secondary structure. The arrangement of the NF-κB dimer within the major groove of one entire turn of DNA gives rise to a global structure that is reminiscent of a butterfly with a DNA ‘body’ and a pair of RHR ‘wings.’ The short, conserved NLS-containing portion of the RHR that is immediately C-terminal to the DD is disordered when present in NF-κB constructs used for X-ray crystal structure determination in complex with DNA (14).

NF-κB recognition of κB DNA at the 5'-end

A set of conserved amino acid residues that are present within a large loop that connects the first two β-strands of the NTD (referred to as loop L1) directly contact bases within κB DNA (63). In the p50 subunit these residues are Arg54, Arg56, Tyr57, Glu60 and His64 (murine amino acid numbering will be used throughout this report) (Figures 4 and 5A). The two Arg, the Tyr and the Glu are each found in all NF-κB subunits. His64, which is conserved in p52 (His62 of human p52) interacts with the 5'-G of the consensus κB DNA sequence. Substitution at this position with Ala in c-Rel, RelA and RelB allows for variance in the identity of this base position and explains why the consensus DNA half-site preferred by these NF-κB subunits is one base shorter than for p50 or p52 (Figure 5B). Consequently, within the context of the biologically relevant NF-κB heterodimers, p50 and p52 subunits preferentially bind a 5 bp long 5'-half site beginning with 5'-GGG while RelA, RelB or c-Rel subunits select for 4 bp half sites that begin 5'-GG. As discussed later, the RelA:RelA homodimer and possibly c-Rel:c-Rel can accommodate 5'-GGGRR if presented with κB DNA

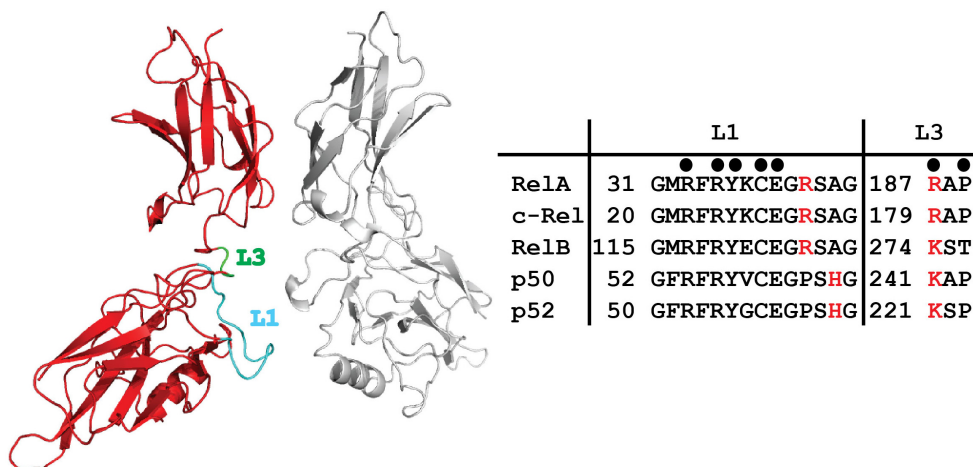


Figure 4. NF- κ B residues that participate in sequence-specific DNA contacts. Left, ribbon diagram representation of RelA homodimer (PDB ID: 2RAM) highlighting loops L1 (cyan) and L3 (green). These two loops contribute all amino acid side chains that contact DNA bases. Right, the amino acid sequences and numbering for loops L1 and L3 are shown for all five murine NF- κ B subunits. Filled circles above mark positions of identically conserved DNA base-contacting residues. Red letters correspond to nonidentical residues that either participate in DNA base-contacts in all cases (denoted by circle) or in specific circumstances, as described in the text.

containing such a half site. A central bp, which is nearly always A:T, is not contacted by either subunit of the NF- κ B dimer. This suggests that p50:p50 or p52:p52 homodimers bind preferentially to an 11 bp κ B DNA composed of two 5 bp half sites separated by a central A:T bp while RelA, RelB and c-Rel select for 9 bp κ B DNA (two 4 bp half sites and a central A:T bp). This model agrees perfectly with the original observation that NF- κ B p50:RelA heterodimer binds to a 10 bp κ B DNA from the enhancer of the immunoglobulin kappa light chain gene (4). The non-contacted central A:T bp provides a convenient reference point for analyzing base-specific interactions by NF- κ B subunits on κ B DNA half sites. For example, the extreme 5'-G that is contacted by the p50 subunit His64 occupies positions at ± 5 bp from this central A:T origin. The G:C bp that occupy positions ± 4 and ± 3 are contacted similarly by identical loop L1 residues in each of the NF- κ B subunits. The two signature arginines (Arg56 and Arg54 in p50) directly contact the two G bases and the invariant glutamate (Glu60 in p50) contacts the paired C base from the complementary strand at the ± 3 positions. Recognition of both nucleotide bases of the G:C bp at the ± 3 position indicates that it plays a more critical role than either of the G:C bp at the ± 5 and ± 4 positions (Figure 5B).

NF- κ B binding to the central bases of κ B DNA

The nucleotide bases at positions ± 2 and ± 1 of κ B DNA vary to a considerably greater degree than the flanking bases at positions ± 3 to ± 5 . In the X-ray crystal structure of NF- κ B p50:RelA heterodimer in complex with κ B DNA from the immunoglobulin kappa light chain gene (14), an arginine residue (Arg187) contained within the flexible loop L3 region of the RelA subunit crosses over to the homologous complementary strand and contacts the T base of an A:T bp at position ± 2 (Figure 5C). The c-Rel subunit also contains Arg at this position (15). The corresponding residue is a lysine in p50 (Lys241 of murine p50), p52 and RelB. The

lysine residue of p50 and p52 can interact either with the complementary T of a ± 2 A:T bp or with a G base from a G:C bp at the same location (16,33). Thus, p50 and p52 can accommodate either A:T or G:C bp at this position. Interestingly, the corresponding lysine in RelB (Lys274 in murine RelB) does not contact DNA. Rather, it engages in an ion pair interaction with the side chain of nearby Asp272. This suggests that RelB is more tolerant than other NF- κ B subunits to DNA base sequence diversity at the ± 2 position of its κ B DNA targets (33,64). This result agrees well with a study reported by Britanova, *et al.*, in which optimal binding sites for RelB:p52 were identified by a random site selection method. The investigators concluded that the preferred RelB:p52 binding sequence mirrors that of the classical p50:RelA consensus, bringing into question the existence of unique RelB:p52 specific binding sites (65).

Base pairs at κ B DNA position ± 1 are not contacted by either NF- κ B RelA or c-Rel subunits (Figure 5C). Lys241 from p50 and Lys221 of murine p52 can contact the ± 1 bp with a preference for A bases (10,11,13). An invariant Tyr on loop L1 (Tyr57 in murine p50 and Tyr36 in murine RelA) crosses over and stacks against the paired bases on complementary strand at both ± 1 and ± 2 positions (63). This mode of interaction is favored by the presence of two successive T bases through contact with their exocyclic 5-methyl groups. Although a Phe at the same position could maintain these stacking interactions, the loop L1 Tyr also mediates hydrogen bonding through its hydroxyl group to a DNA backbone phosphate making Tyr the preferred residue for recognition and binding of the central bases. Two C bases or a T and C can also accommodate binding by the loop L1 Tyr, but an A or G base at either position is unfavorable.

The importance of this Tyr in κ B DNA sequence selection is illustrated by the popularity of the sequence AAATT or AATTT (the central bp is underlined) at the central 5 positions in κ B DNA targeted by RelA and c-Rel homodimers. It seems likely that the favorable interaction between Tyr and neighboring pyrimidine bases near the center of the

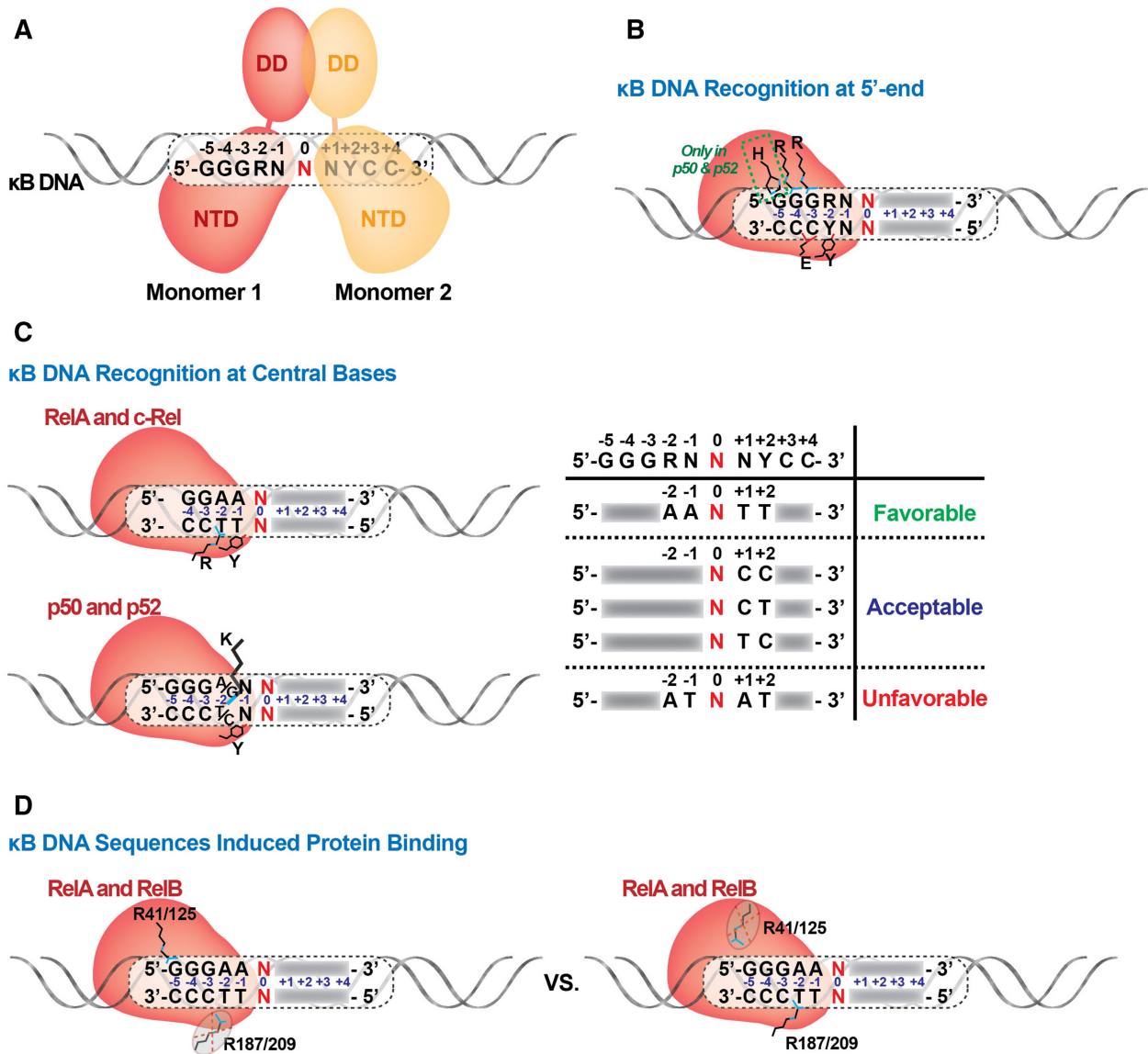


Figure 5. Variable modes of NF-κB:DNA recognition. (A) NF-κB binds to κB DNA as a dimer and each monomer recognizes one half site. (B) Standard recognition of κB DNA at the 5'-end by NF-κB. The conserved NF-κB subunit loop L1 residues involved in direct contacts with DNA bases are indicated. Arg35 (murine RelA numbering) contacts the G base at the -4 position and Arg33 contacts the G at -3. His64 or His62 are specific only to p50 and p52 subunits, respectively. (C) NF-κB recognition of κB DNA at the central bases. Left, different modes of DNA binding (for RelA and c-Rel vs. p50 and p52) are shown. Right, table summarizing how the presence of specific nucleotides at certain central positions affect NF-κB:DNA binding. (D) κB DNA sequence induces NF-κB binding with distinct modes. R41/R125 of RelA/RelB binds the G:C bp at -5 position of a 5 bp half-site. R187 of RelA cannot bind the -2 position of the same half-site.

9 bp κB sites compensate for the fact that NF-κB RelA and c-Rel subunits each contact fewer flanking GC bp than either p50 or p52. The importance of two neighboring pyrimidines at the complementary strand ±1 and ±2 positions is further reinforced by the observation that they are present in at least one half site of all functional κB sites known to date, even if the second half site exhibits significant degeneracy. By contrast, otherwise ideal κB DNA sequences that lack neighboring pyrimidine bases at these positions, such as GGGATAATCC and GGGATTATCC (central bp is underlined), fail as functional κB sites.

It is not clearly evident from the structural studies why position 0 is dominated by AT bp. It seems likely that this

might be necessary to convey the proper DNA bending and/or dynamic characteristics (discussed later) required for optimum NF-κB:DNA complex formation. Interestingly, despite the pseudo-symmetry in κB sites with identical half sites, NF-κB homodimers bind DNA asymmetrically. This is clearly demonstrated in the co-crystal structure of p52:p52 homodimer bound to a κB DNA of sequence GGGGATTCCCC (13). This DNA contains two identical half sites and only the central base-pair (underlined) breaks the otherwise perfect symmetry. However, one p52 subunit from the homodimer participates in many more DNA ribose-phosphate backbone contacts than the other. Non-uniformity in DNA bending and resultant asymmet-

rical positioning of the NTD on different DNA half sites might explain the asymmetry of the two half complexes.

κ B DNA sequence induces distinct NF- κ B binding modes

While the initial X-ray crystal structures together with accompanying *in vitro* and cellular biochemical studies supported DNA binding according to the rules described above, subsequent structural analyses revealed that alternate binding modes are available to RelA and RelB (33,58,66). As previously discussed, both RelA and RelB bind preferentially to 4 bp 5'-GGAA-3' type half-sites. However, slight rearrangement of side chain geometries together with modest movement of the entire, folded N-terminal domain allows both of these NF- κ B subunits to also bind a 5 bp 5'-GGGAA-3' half-site (Figure 5D). In other words, like the p50 and p52 subunits, RelA and RelB subunits are capable of contacting the ± 5 G in spite of their lack of a histidine at the position equivalent to His64 of p50 (16,33). This is accomplished when a nearby arginine (Arg41 of RelA and Arg125 of RelB) alters its conformation to contact the guanine at position ± 5 . However, this does not occur without disrupting the ability of the inter-domain linker arginine residue (Arg187 of RelA) to contact the A:T bp at ± 2 positions in all 4 bp half sites. The switch by RelA and RelB to binding outer versus central bp in a 5 bp half site is probably the result of three factors: the differential strength of the contact between Arg41/125 and G-5 and between Arg187/Lys274 and T-2 of A:T, structural difference between 5'-SGGAN-3' (S = C, T or A) versus 5'-GGGAN-3' half sites on account of relative dynamic behavior as discussed later, and the preferences for the adoption of preferred conformations by the protein side chains involved. Therefore, NF- κ B proteins have evolved alternative stable conformations that allow for binding of a variety of DNA sites by accommodating DNA sequence and conformational variations. Although it has not yet been captured in X-ray crystal structures, we expect c-Rel to be capable of similar alterations in conformation in order to accommodate binding to different classes of κ B DNA.

Stabilization of NF- κ B:DNA complexes through NF- κ B subunit inter-domain interactions

It is well established that amino acid residues located far from the interaction surface of a protein can contribute indirectly to modulate a binding interface and NF- κ B is not an exception (67). This becomes clear upon comparison of DNA binding by homologous pairs of NF- κ B homodimers such as p50:p50 and p52:p52 or RelA:RelA and c-Rel:c-Rel. In both pairs, all DNA contacting residues are identical. However, each dimer binds DNA in a unique manner. *In vitro* selection experiments have revealed a preference for each NF- κ B homodimer toward a subset of κ B sites (68). It is difficult, however, to pinpoint precisely which distal residues affect binding or by what mechanisms they exert their influence over DNA sequence selectivity.

Additional cellular factors that interact with NF- κ B can have a profound effect on DNA recognition and binding,

even when the site of interaction with NF- κ B is far from its DNA binding surfaces. The effect is mutual, as NF- κ B binding to DNA can also influence distal NF- κ B:protein interactions. This suggests that the assembly of NF- κ B into large multiprotein enhanceosome complexes can be either facilitated or inhibited by subtle changes in DNA conformation. One mechanism by which co-factor interaction could directly influence DNA binding affinity is illustrated by the interplay of two loops within the DNA binding RHR of NF- κ B subunits: one from the DD and the other from the NTD. The loop that connects β -strands f and g in the NF- κ B DD (the ' β f- β g loop') projects toward κ B DNA but does not directly contact it (63). Two conserved acidic residues (Asp267 and Glu269 in chicken c-Rel) are located within this loop and reside near the DNA in the complex between c-Rel:c-Rel homodimer and the IL2-CD28RE κ B DNA complex. Although these residues do not directly contact DNA, their proximity and combined electrostatic surface potential likely repel DNA and weaken binding (15). These negative charges are neutralized, however, by one of the Arg residues from loop L1. Loop L1 is the same loop that contributes five of the six base contacting residues. Loop L1 can be divided into three parts: N-terminal front, N-terminal back, and the C-terminal portion. The C-terminal portion of loop L1 is flexible and can contact the DNA backbone of nucleotides flanking the κ B sequence. The N-terminal front and back, on the other hand, adopt stable structures that remain unchanged both in DNA bound and unbound states (15). Surface residues from the front portion contribute the DNA base-contacting residues. It is an Arg from the back surface of this ordered N-terminal portion of loop L1 that contacts the acidic residues of the β f- β g loop. Interestingly, although these residues are conserved across NF- κ B family subunit proteins, not all NF- κ B:DNA complex crystal structures exhibit this interaction. We suggest that DNA conformational differences play a role in dictating RHR inter-domain interactions that serve either to augment or weaken DNA binding affinity. In the case of oncogenic v-Rel, a viral homologue of c-Rel from the avian Reticuloendotheliosis virus, two core residues within the rigid part of loop L1 are mutated. Mutation of these two residues is at least partly responsible for the observed altered DNA binding profiles by v-Rel as compared to c-Rel (69). Finally, the β f- β g and L1 loops contain sites for post-translational modification, which also regulates NF- κ B:DNA recognition as discussed below. Taken together, these observations suggest that subtle changes to the interactions between domains of the RHR, either as a direct result of binding to proteins or indirectly via factors that modulate target DNA conformation, serve to influence NF- κ B:DNA complex stability.

The development and implementation of high throughput techniques to study how transcription factors bind to target DNA sequences have been extraordinarily useful in providing new insights to the field of DNA binding by NF- κ B. Some of these approaches include SELEX (systematic evolution of ligands by exponential enrichment), PBM (protein-binding microarrays), and EMSA-Seq. Such

genome-wide analyses have led to the identification of a plethora of new non-consensus NF- κ B DNA binding motifs as well as serving to classify NF- κ B binding sites, including those that contain only a single consensus κ B half-site, revealing the intrinsic plasticity of NF- κ B dimers (64,70). In general, results from these approaches agree very well with data obtained through structural and *in vitro* biochemical methods.

DNA CONFORMATION AND NF- κ B BINDING

Sequence-dependent DNA conformations

DNA duplexes are not static entities that simply present themselves to proteins to be read and then seed assembly of multiprotein complexes at specific sequences. Rather, in solution DNA is intrinsically dynamic on many levels and time scales. The movement of DNA through its different conformational states is continuous and is influenced by, but not completely dependent upon, its nucleotide sequence. Nuclear magnetic resonance (NMR) and X-ray crystal structures of different κ B DNA (21,71–72) together with molecular dynamics simulations of DNA in its unbound state (73) have revealed insights that highlight the complexity of the NF- κ B:DNA recognition process.

NMR solution studies of free κ B DNA from the long terminal repeat (LTR) of proviral DNA encoding HIV-1 (HIV- κ B DNA) as well as two different mutant versions of the same sequence revealed that phosphate backbone dynamics vary not only between different contiguous base-pairs but also between the same contiguous base-pairs within different contexts (71,72). This complicated backbone dynamic dictates local major and minor groove depth and width as well as subtle differences in base functional group location and geometry. For example, it has been observed that the sequence of nucleotides that flank a κ B site impact the overall groove chemistry of the κ B site in ways that affect p50:RelA heterodimer binding (44,74). The significance of flanking sequences in modulating DNA target site structure and transcription factor (TF) binding has also been reported for other systems such as the glucocorticoid receptor (GR) (75).

Microsecond time-scale molecular dynamics (MD) simulations of a 20 bp long DNA fragment of the IL-2 promoter containing the IL-2 κ B site revealed two striking features: the -2 position A nucleotide of the IL-2 κ B site (AGAAATTCC) begins intercalative stacking with the cross-strand bases, disrupting the base pairing of the central A:T base pair flipping out of the DNA helix (73) (Figure 6). In light of what is known about interactions that stabilize NF- κ B:DNA complexes, these massive structural variations in a κ B site must affect NF- κ B binding. Although we do not know precisely how DNA conformations impact NF- κ B binding, it is likely that the AT-rich sequence typical within central portion of κ B DNA facilitates both cross-strand base stacking and base-flipping processes. Many κ B sites that favor binding to RelA:RelA or c-Rel:c-Rel contain four or five A:T bp at their center. It is tempting to speculate that these DNA rely upon the dynamic inherent to such sequences either to facilitate initial binding by these dimers or to hinder binding by other dimers.

IL2- κ B DNA

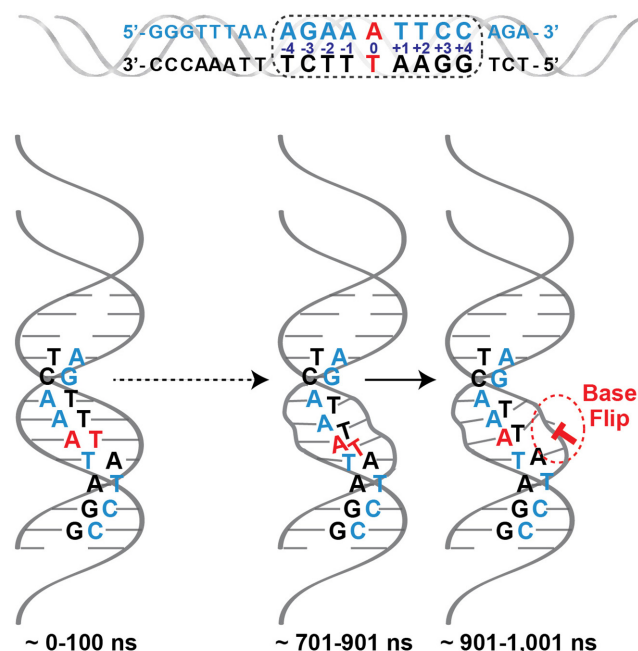


Figure 6. Molecular dynamics (MD) of IL-2 κ B DNA in its unbound state. Upper panel, the 20 bp DNA sequence from the promoter for IL-2 is depicted with its consensus κ B DNA site enclosed in the dashed outline box. Bottom panel, a cartoon representation of changes that occur consistently during the time course of the MD simulation within the central A:T portion the IL-2 κ B DNA that lead to flipping of the 0 position T base. Time ranges are denoted in nanoseconds (ns).

Single nucleotide changes in κ B DNA can alter gene expression

One of the fascinating observations involving gene regulation by NF- κ B is the ability of a particular NF- κ B dimer to initiate different gene expression programs upon binding κ B sites that differ at only a single bp. This phenomenon was first described by Leung *et al.* in the Baltimore Laboratory where the authors showed that the RelA:RelA homodimer activates transcription of genes within a promoter containing the κ B DNA sequence GGGAAATTCC but represses gene transcription when the κ B DNA sequence is GGGAAATTCC (76). These two κ B DNA differ only by a single bp (A:T to T:A) at the 0 position. RelA:RelA homodimer is recruited to the two sites and binds to both sequences with similar affinity both *in vivo* and *in vitro*. As previously discussed, the central bp that separates the half-sites has not been observed to participate in contacts with protein in any NF- κ B:DNA complex structure studied. How such a small, seemingly inconsequential change in sequence might lead to such severe functional differences remains unclear. However, the importance of seemingly minor variations in κ B DNA sequence to transcriptional regulation has since been observed in other NF- κ B dimers. In 2012, researchers observed that p52:p52 homodimer could differentiate between A:T and G:C bp at the central position (48). In both instances, the p52:p52 binds its DNA target but only in the case of the central G:C bp can the ho-

modimer also associate with its protein co-regulator Bcl-3 and activate transcription by recruiting histone acetyltransferases. When the central bp is A:T, the same p52:p52:Bcl-3 complex represses gene transcription via recruitment of histone deacetylases. Since most κ B sites include A/T-centric κ B sites, the p52:p52:Bcl-3 complex down regulates most genes that are preferentially activated by RelA dimers. On the other hand, the p52:p52:Bcl-3 complex appears to be the primary NF- κ B activator for a smaller group of genes that contain G/C-centric κ B sites within their promoters. RelA-containing dimers bind poorly to these G/C-centric κ B sites. Since most NF- κ B responsive promoters include multiple κ B sites, selection of these κ B sites will be guided generally by the relative availability of specific NF- κ B dimers and the affinities of the respective NF- κ B:DNA complexes. But, as these studies on the functional consequences of single nucleotide changes attest, select κ B DNA sequences can specialize in the recruitment of particular NF- κ B dimers with specific transcriptional outcomes.

NF- κ B binds to κ B DNA with only one half-site consensus

The mutually semi-independent, two-domain modular architecture of the NF- κ B subunit RHR endows it with potential to accommodate expanded target DNA sequences. As described previously, both the NTD and DD participate in contacting DNA, although the DD does so nonspecifically through contacts with the DNA phosphate-ribose backbone only. The two domains are linked by a short stretch of roughly 10 residues. Due to the fact that NF- κ B dimerization is mediated by relatively stable association between the DD from two subunits, the resultant NF- κ B dimers possess three stable structural entities: two NTDs and one platform composed of two DDs. The flexibility of the NTDs relative to the DD platform allows them to move upon encountering non-ideal binding half-sites in order to locate optimal binding surfaces as first shown by Chen *et al.* (57). If necessary, the NTD can even move so far on a half-site as to interact only with the DNA phosphate-ribose backbone, abandoning any base-specific interactions (57,77) (Figure 7). Consequently, κ B DNA that contain only one half-site consensus can serve as NF- κ B dimer binding targets of reasonable affinity (61). In these cases, the NTD from one subunit recognizes one consensus DNA half-site with specificity making all possible base contacts while the other contributes positively to complex stability by moving into a position that fails to contact any nucleotide bases directly but allows for non-specific interactions with the DNA backbone. Recent *in vivo* and *in vitro* results have confirmed the existence of κ B sites with only half-site specificity (56,64,77).

Interestingly, in these cases of NF- κ B binding to κ B DNA that bear only one consensus half-site the non-specific half-site sequence cannot be random. As discussed previously, the stacking of a Tyr against preferred TT bases on that complementary DNA strand at both ± 1 and ± 2 positions contributes both specificity and affinity to NF- κ B:DNA complexes. Therefore, a non-specific half-site with TT in positions +1 and +2, as in GGGAA A/T TTNN, is favored. Moreover, non-specific half-site sequences with stretches of G or A (for example, GGGAA A/T GGGG

or GGGAA A/T AAAAA) are not preferred (Figure 7). As discussed in a previous section, DNA base sequences outside the border of the κ B DNA consensus can indirectly influence NF- κ B binding by altering the conformation and dynamics of the non-specific half-site. A similar mode of binding is observed in tetrameric p53 where the tetramerization domain is flexibly linked to the DNA binding domain. In that system it is observed that one p53 dimer binds DNA with sequence specificity whereas the other dimer binds non-specifically with alternative conformation (78,79). It seems likely that many, if not most, multimeric TFs with multidomain modular structures can accommodate expanded DNA sequences through optimized half-site binding.

NF- κ B dimers bind to tandem κ B sites

All gene promoters contain binding sites for many transcription factors and often promoters contain multiple binding sites for a single transcription factor family. It was initially proposed that multiple transcription factors bind to their respective sites cooperatively and activate transcription synergistically (80–82). In support of this notion, researchers measured increased levels of reporter gene expression by multiple transcription factors in overexpression systems. In fact, synergistic transcriptional activation through cooperative interactions of transcription factors has been found to be the case in only some instances. In such cases, although cooperative interaction between two transcription factors bound to tandem sites occurs, it is mostly, if not exclusively, the result of two monomeric transcription factors or else a dimer and a monomer binding to a composite DNA response element (83–85). Other studies have also highlighted that the DNA sequence has an active role in determining how TFs bind and act cooperatively to regulate gene expression (86,87). Along these lines, it has been suggested that when two transcription factor binding sites are within sufficiently close proximity, their corresponding flanking regions can overlap and, as a consequence, their final sequences might differ considerably from the expected consensus. Such composite sites would be expected to display relatively low affinity toward their respective transcription factors individually, suggestive of the crucial role of the DNA sequence in transcription factor cooperativity. NF- κ B dimers have never been shown conclusively to interact cooperatively with other transcription factors bound to tandem sites. One recent report described an approach to determine cooperative interaction between a large number of TF pairs through the use of selected DNA (86). When the NF- κ B p50 subunit was used in this *in vitro* selection-based method, only p50 itself could be identified as a partner protein.

The most extensively studied system for measuring cooperativity in NF- κ B-driven gene expression is the interferon beta (IFN- β) gene promoter, which contains a high affinity κ B site adjacent to two IFN-stimulated response element (ISRE) binding sites for transcription factor IRF3/7 (88). Although both TFs bind independently to their respective sites, direct protein-protein interaction between NF- κ B the RelA homodimer or p50:RelA heterodimer and IRF3 that facilitates their DNA binding has never been convinc-

Half-site Consensus

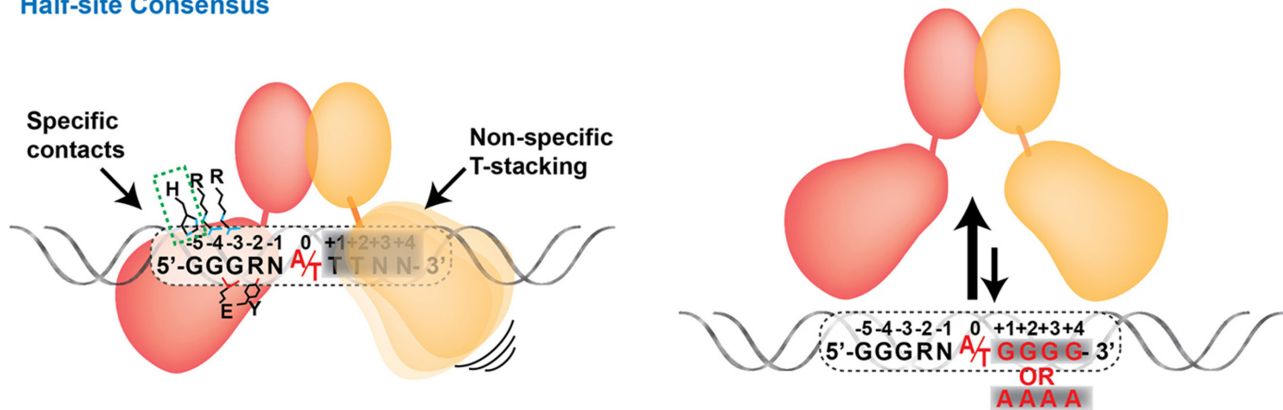


Figure 7. NF- κ B dimer binding to κ B sites with only half-site consensus. Left, one monomer makes all specific possible contacts whereas the other monomer fails to specifically contact any nucleotide base. Right, when the non-consensus κ B DNA half site includes either GGGG or AAAA, the NF- κ B:DNA complex formation is strongly disfavored.

ingly shown (89). Using solution-based experiments, however, Dragan, *et al.*, were able to observe cooperative binding interactions between the p50 homodimer, IRF3, and DNA (90). In addition to κ B and ISRE, the IFN- β promoter also contains a binding site for the AP1 transcription factor family member ATF2/c-Jun. Although attempts have been made to assemble all these transcription factors on an IFN- β promoter fragment *in vitro*, a stable assembled complex containing all proteins could not be formed (62).

Several NF- κ B response genes contain two or more κ B sites within their promoters or enhancers where the arrangements of the κ B sites do not follow any specific spacing or orientation. The most extensively studied of these promoters is the HIV LTR where two identical κ B sites are present with an intervening spacing of 4 bp. It was shown nearly 30 years ago that NF- κ B dimers do not bind these sites cooperatively. Yet, they do synergistically activate reporter gene transcription (91,92). That is to say, together the two κ B sites drive reporter gene expression at levels higher than the sum of each individual site. Indeed, one study suggested a cooperative binding model based on their observation that two dimers can occupy both sites simultaneously (92). *In vitro* biochemical experiments suggested an anti-cooperative relationship of binding between the two NF- κ B dimers for tandem κ B sites (61). It was further showed that expansion beyond two κ B sites can further increase levels of reporter gene expression and that the maximum transcriptional threshold is achieved by four tandemly arranged κ B sites (92). A relatively recent report examined the relationship between NF- κ B concentration, gene expression, and number of κ B sites within the regulatory element. They found gene expression was gradual and increased as a function of number of κ B sites (93).

The apparent lack of cooperativity between NF- κ B dimers in binding to tandem κ B sites makes sense since, due to the variability in spacing between pairs of κ B sites observed in gene promoters, oriented physical interaction between the dimers bound to neighboring sites is likely not possible. There have been two X-ray crystal structures reported that capture a pair of NF- κ B dimers bound to DNA containing tandem κ B sites. One contains two NF-

κ B p50:RelA heterodimers bound to tandem HIV- κ B sites and the other includes two NF- κ B RelA:RelA homodimers bound to tandem E-Selectin κ B sites (66,94). Biochemical experiments revealed that binding of one dimer does not facilitate binding of the second dimer (61). Rather, the *in vitro* study indicates that binding affinity of a second NF- κ B dimer is weakened by the presence of a first bound dimer. In other words, assembly of the dimers on tandem κ B sites is anti-cooperative (Figure 8). This is in spite of the fact that there are direct contacts between the two dimers bound to DNA (66,94). Interestingly, although the two κ B sites in each DNA fragment are identical in sequence, the complexes form asymmetrically. For example, the RelA:RelA homodimer:E-Selectin κ B DNA complex reveals a unique binding mode in which one of the dimers binds its respective κ B site suboptimally with several DNA contacts missing. The authors speculate that this mode of binding represents an early step in the process of NF- κ B dissociation from DNA (66).

If NF- κ B dimers on promoters with close tandem κ B sites do not cooperate with one another during the assembly process, and if this is even less likely to be the case when the sites are separated by tens of bp, then why do so many target genes possess multiple NF- κ B binding sites? We envision two possible consequences of multiple κ B sites. First, a promoter with multiple κ B sites increases its probability of successfully recruiting NF- κ B. While this at first seems obvious, it raises the intriguing possibility that the binding event, and not necessarily the stable complex, is key to transcriptional activation. Were this the case, then a mechanism that raises the number individual binding events over time would serve to increase overall transcriptional output (Figure 8). The second advantage is that the different κ B sites could be NF- κ B family member-specific. As explained previously, RelA:RelA homodimer binds poorly to a G/C-centric DNA but with high affinity to A/T-centric DNA, whereas the p52:p52 homodimer in complex with Bcl-3 binds both classes of DNA but with opposite transcriptional outcomes. Thus by simply exchanging dimers, transcriptional output can be prolonged or tempered (95). Under this model, the addition of a particular κ B site within

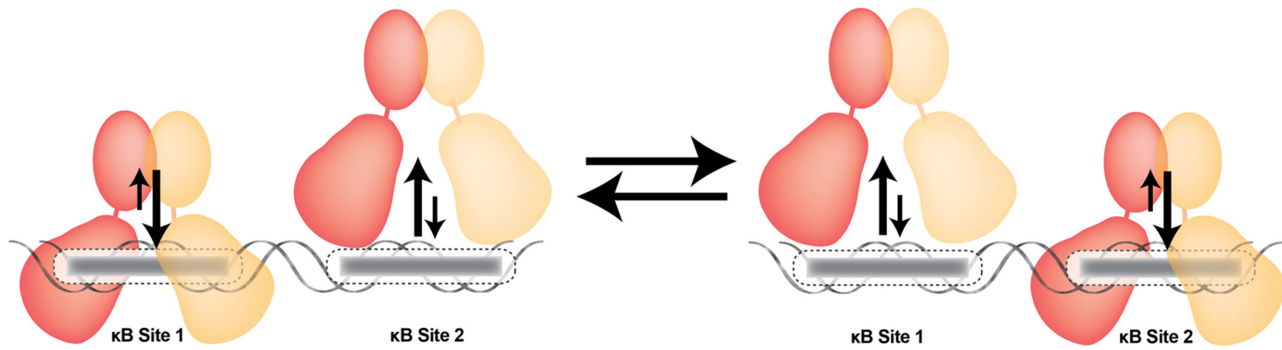


Figure 8. NF- κ B dimers binding to tandem κ B sites. Anti-cooperative binding of two NF- κ B dimers to tandem κ B sites. Only one dimer can stably bind to one site at any given time.

a target gene promoter would expand inducible expression of that gene to the specific class of NF- κ B that functions through that site and the signaling pathway that mobilizes the corresponding NF- κ B.

ALLOSTERIC REGULATION OF NF- κ B BINDING TO DNA

Over the past few years, genome-wide experiments have raised some intriguing observations surrounding DNA binding by transcriptional regulatory factors. Among these, that in addition to binding their own consensus sites, most transcriptional activator proteins also bind at places in which there is no consensus site present (96,97). In separate studies, transcription factors have been studied by introducing mutations that target their DNA interacting residues and render them incapable of binding to DNA response elements (98). Surprisingly, when such mutants are then tested for their transcriptional potential, many are still able to engage in promoter-specific binding events and elevate gene expression (99,100). Overall, observations from these studies suggest that a typical transcription factor is able to regulate gene expression in two ways: by directly binding to their cognate DNA response elements, and by associating with DNA indirectly via interaction with another DNA-bound factor. Due to this second mode of action, DNA binding by the transcription factor is not required and, hence, mutations that abolish its DNA binding or even the deletion of the DNA binding domain entirely do not necessarily affect its ability to regulate gene expression (76,101).

As is the case with other transcription factors, NF- κ B RelA-containing dimers have been shown to regulate gene expression through both direct and indirect interaction with promoter DNA. The observation that NF- κ B could regulate gene expression through an indirect mechanism arose from a genome-wide study reported 15 years ago (54). This study found that nearly one third of RelA genome reading events could not result from direct interaction with DNA since κ B sites were absent from those genes. Several subsequent reports have validated this earlier observation and found that NF- κ B associates with many promoter/enhancer elements that lack any κ B site (55). Consensus binding sites for other transcription factors are often present within the DNA where NF- κ B is bound in these cases. The most well studied example came from a

RelA ChIP experiment targeting a promoter that contains ISRE (interferon-sensitive response element) sites but not κ B sites. The ISRE recruits IRF3/7 transcription factors through direct binding, while NF- κ B is tethered indirectly to the DNA via its interaction with IRF3/7. The reciprocal case has also been reported wherein NF- κ B was identified bound to a κ B site and IRF3 associated with the same DNA indirectly via binding through the NF- κ B RelA subunit (101,102). The following sections discuss how additional factors can tether themselves to DNA via NF- κ B and why this is an essential component of NF- κ B-dependent gene expression.

DNA binding by NF- κ B is influenced by protein co-factors

In addition to IRF3, many other factors have been reported to interact with the RelA subunit when NF- κ B is bound directly to κ B DNA. Many of these 'other factors' are themselves DNA binding transcription factors, such as p53, E2F1, FOXM1 and KLF-6 (53,55,103–105). However, several additional factors that are not recognized as TFs, such as the DNA repair protein OGG1, architectural DNA binding protein HMGA1 (formerly HMGI(Y)), RNA binding proteins such as RPS3 and Sam68, and the histone chaperone protein nucleophosmin/NPM1, have also been reported to engage with NF- κ B on promoters of target genes (106–109). Gene knockout or knockdown studies have revealed that each of these proteins plays critical roles in gene expression via RelA. However, their effect is selective and only a subset of RelA-regulated genes is influenced by each of these proteins. *In vitro* experiments have shown that p53, FOXM1, RPS3, Sam68, and NPM1 facilitate DNA binding of RelA dimers (103,106,108,110). These factors interact directly with RelA, often through its DNA binding RHR segment. Although never quantitatively measured, the affinity of interaction between NF- κ B and these factors appears to be weak, since their complexes have not been successfully isolated by size exclusion chromatography though their interaction *in vitro* or in cells has been confirmed by co-immunoprecipitation or other affinity-based pull-down methods. Such protein:protein interactions are not unprecedented in the literature, where it has been reported that many micromolar and even some nanomolar affinity complexes that are readily identifiable via immunodetection fail to co-purify in the time scale require-

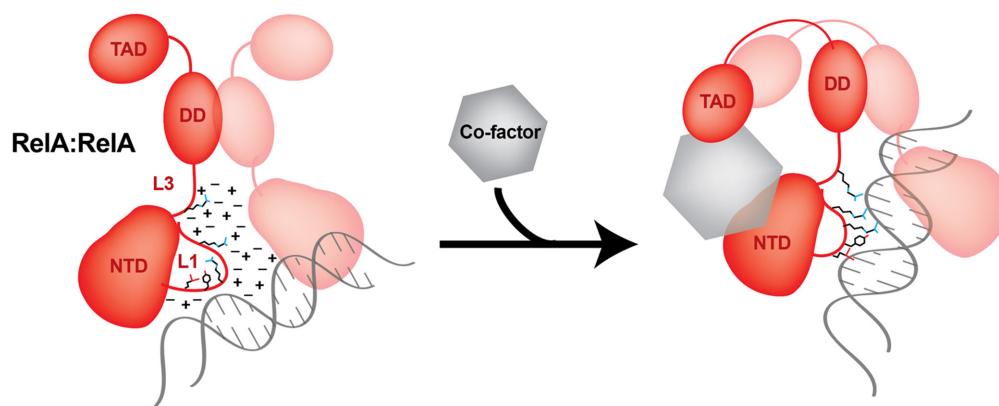


Figure 9. A model of how co-factor stabilizes NF- κ B:DNA complex. At physiological ionic strength, ions shield DNA contacting residues along their variable conformational flexibility. Upon binding to a co-factor, dissolved ions may lose some of their ability to shield DNA and/or RelA side-chain conformations might alter themselves to a mode primed to bind DNA.

ments of size exclusion chromatography (111,112). We refer to these protein factors as ‘co-factors’ of NF- κ B. The increased DNA binding affinity exhibited by NF- κ B in the presence of protein co-factors has been described as site-specific since ChIP showed that NF- κ B and co-factors assemble on specific promoters in response to specific stimuli (113). *In vitro* binding studies concurred with *in vivo* experiments for many of these proteins and in all cases tested it was shown that the co-factors strengthened NF- κ B binding to κ B sites (53,108,114,115). Interaction of p53 and FOXM1 with p50:RelA heterodimer was confirmed both in cells and *in vitro* (103).

It has long been observed that NF- κ B dimers do not efficiently bind to κ B DNA under physiological ionic strength (116–119). High ion concentrations effectively shield charged/polar groups on the surface of RelA, p50, and DNA and decrease the strength of their interactions. However, in the presence of RPS3 and, to a lesser extent, p53, RelA dimers recover the ability to binding to κ B DNA with high affinity (119). Although the precise mechanism for how RPS3 and other co-factors accomplish this is unclear, we can speculate that RPS3 mobilizes or otherwise occupies ions, mitigating their shielding effect near the NF- κ B DNA binding surfaces. Furthermore, low affinity but direct contacts between RPS3 and RelA could induce long-range allosteric changes in DNA contacting residues of RelA such that these residues can participate in base-specific contacts with DNA (Figure 9). Protein flexibility test has shown that the segments of both p50 and RelA far away from the DNA binding surface become more flexible upon binding to a κ B DNA (120). These distal allosteric sites might be the targets of co-factors affecting DNA binding. Whatever the mechanism, it is clear that diverse protein co-factors function to negate the detrimental ion effect and facilitate formation of NF- κ B RelA:DNA complexes. Since many, if not all DNA binding transcription factors exhibit decreased DNA binding as a result of increased ion concentration, as a general rule they all likely require co-factors to facilitate their binding.

The observation that many different proteins can function in the nucleus as co-factors to modulate transcriptional activity sheds light on some previously studied interactions between NF- κ B and other transcription factors. For example, nuclear hormone receptors, in particular glucocorticoid receptor (GR), are known to inhibit NF- κ B activity, consequently behaving as anti-inflammatory agents. Although several mechanisms have been invoked to explain the anti-inflammatory activity of GR, the most critical is likely its ability to block transcriptional activity of RelA-containing NF- κ B dimers on DNA (99,101). Upon binding to glucocorticoids, GR can inhibit the effect of co-factor interaction with RelA by removing it from its DNA binding transcription factor partner IRF3, as GR and IRF3 appear to bind RelA through a common surface. In its glucocorticoid-bound state, GR can remove RelA from IRF3 at ISRE sites. Furthermore, GR can suppress inflammation by binding RelA upon κ B sites within the promoters of pro-inflammatory target genes. In contrast to its removal of RelA from DNA-bound IRF3, GR inhibits NF- κ B-dependent target gene expression without disrupting the RelA:DNA complex. Exactly how GR association inhibits the transcriptional activation potential of RelA even when a RelA-containing NF- κ B dimer remains bound to DNA is at present unknown and open to speculation. As mentioned previously, merely binding to DNA does not necessarily give a transcription factor license to activate gene transcription. It is possible that GR binding alters the kinetic rates with which RelA-containing NF- κ B dimers associate with and release DNA. If particular DNA binding kinetics were linked to downstream gene regulatory events such as co-activator or co-repressor recruitment, then one could envision how disruption of this timing could alter the transcriptional output without necessarily removing the DNA binding protein. Such kinetic control over enzyme catalytic mechanisms has been proven an effective mechanism employed by many binding protein enzyme inhibitors, such as INK proteins toward cyclin-dependent kinases (Cdk) in cell cycle regulation (121). Alternatively, GR can directly

block RelA recruitment of activating components such as p300/CBP or other co-activators independent of its effect on DNA binding kinetics.

Co-factors can influence DNA conformation

Not all co-factors that affect NF- κ B binding to DNA do so through direct interactions with NF- κ B. The DNA repair protein OGG1, for example, binds to a damaged site (O⁶-methyl guanine) somewhat distal to the κ B site, which is essential for NF- κ B recruitment (107). It is likely that upon binding to the damaged DNA, OGG1 alters the structure of the κ B site (Figure 10A). In the process, the location and/or context of the modified base are critical. *In vitro* experiments suggest that modification of G residues in specific positions lead to more efficient recruitment of NF- κ B (107). This is another area in which mechanistic details remain to be worked out, but it is possible that both linear distance and relative orientation of the modified base influence its ability to regulate recruitment of NF- κ B to κ B sites.

Architectural transcription factors can also enhance recruitment of RelA dimer to κ B sites (Figure 10B). HMGA1, an AT hook binding protein, was shown to increase promoter activity of IFN- β (109). The IFN- β promoter contains multiple HMGA1 binding sites, one of which overlaps with the κ B site (IFN β - κ B). IFN β - κ B site contains five A:T bp in the central region, which is also the binding site for HMGA1. Interestingly, both HMGA1 and NF- κ B are proposed to bind the same κ B site with HMGA1 lining the minor groove side while NF- κ B contacts DNA bases through one turn of the major groove. However, on the basis of X-ray crystal structure analysis, it is not possible for both proteins bind to a single κ B site simultaneously without significant structural rearrangement. Although the positive role of HMGA1 in NF- κ B-mediated gene regulation *in vivo* is unquestioned, the mechanism of how it facilitates the formation of NF- κ B:DNA complexes remains unresolved.

The role of HMGA1 in activation of the immunoglobulin heavy chain enhancer μ during B cell development has been investigated thoroughly (122). HMGA1 displays strong interactions with PU.1 and Ets-1 transcription factors and binds weakly with DNA. These interactions have been proven critical for μ chain gene enhancer activation. Based on these and other experiments, we propose the following model for HMGA1 enhancement of NF- κ B dimer binding to DNA. HMGA1 transiently binds through the minor groove at AT-rich sites altering the conformation of the DNA major groove and allowing NF- κ B dimers to initiate binding from major groove side. HMGA1 then dissociates upon binding of NF- κ B dimers to κ B DNA (Figure 10B). In this model the HMGA1 protein acts more as a 'catalyst' to accelerate proper NF- κ B:DNA complex formation. Several reports also suggest that HMG proteins compete with Histone H1 protein and that, through competitive removal of H1, HMG proteins cause chromatin decompaction facilitating transcription factor access to their DNA response elements (123). Thus, HMGA1 can facilitate DNA binding of NF- κ B in at least two ways: by improving access to DNA binding sites and by priming the κ B DNA conformation for more efficient NF- κ B binding.

The transcription activation domain influences RelA binding to DNA

All structural and most other biophysical characterization of NF- κ B:DNA complexes has been performed using the DNA binding RHR. It was believed that the acidic, C-terminal transcription activation domain (TAD) of RelA functions independently in solution and, perhaps, might negatively affect DNA binding through non-specific charge-change interactions between the basic RHR and acidic TAD. Interestingly, early experiments revealed full length RelA that was produced in an *in vitro* transcription-translation system failed to bind κ B DNA. However, deletion of the TAD resulted in enhanced DNA binding by the RelA RHR (124). We have subsequently reproduced those original results and further demonstrated that an independently expressed RelA TAD could act as an inhibitor preventing the RHR from binding to κ B DNA (66).

The biophysical and biochemical properties of the RelA TAD have been studied extensively. The RelA TAD can be divided into two parts, TA1 and TA2, based on their ability to interact with partner proteins. The more C-terminal TA1 interacts with Mediator and with components of the basal transcription machinery (125–127). The more N-terminal TA2 binds the CBP/p300 co-activator (Figure 1A). In addition, the KIX domain of CBP/p300 was shown to contact the RelA RHR through a binding interaction that is dependent upon phosphorylation at RelA residue Ser276 (128). Overall, these observations suggested a general mechanism in which CBP/p300 facilitates RelA binding to DNA by neutralizing the inhibitory effect of the highly acidic TAD. However, more recent solution-based experiments reveal that the TAD does not prevent DNA binding by RelA under conditions that approximate physiological cellular environment, even in the absence of co-factors (119). Furthermore, recombinant full length RelA retains its ability to bind DNA, albeit with lower affinity, under high salt conditions that fail to support DNA binding by the RelA RHR alone. The weak binding exhibited by full length RelA increases significantly in the presence of protein co-factors while, surprisingly, most co-factors tested, RPS3 and p53, fail to influence DNA binding by the same RelA in the absence of its TAD (119) (Figure 9). Therefore, the RelA TAD appears to play a fundamental role in DNA binding under physiological conditions. Precisely, how the interplay between the well characterized DNA binding RHR, the complicated regulatory potential of the TAD, and the subtle facilitative nature of protein co-factors results in κ B DNA binding and target gene expression remains unclear at this time. It is possible that it influences phase separation and formation of nuclear condensates as has been described recently for other transcription factors such as OCT4 and GCN4 (129). However, this is an intriguing area of investigation that is expected to unravel critical insights into the mechanism of NF- κ B:DNA complex formation.

Post-translational modifications affect DNA binding by NF- κ B

Several recent reports have shown that methylation and acetylation of arginine and lysine residues within or immediately after RelA RHR modulate gene expression by alter-

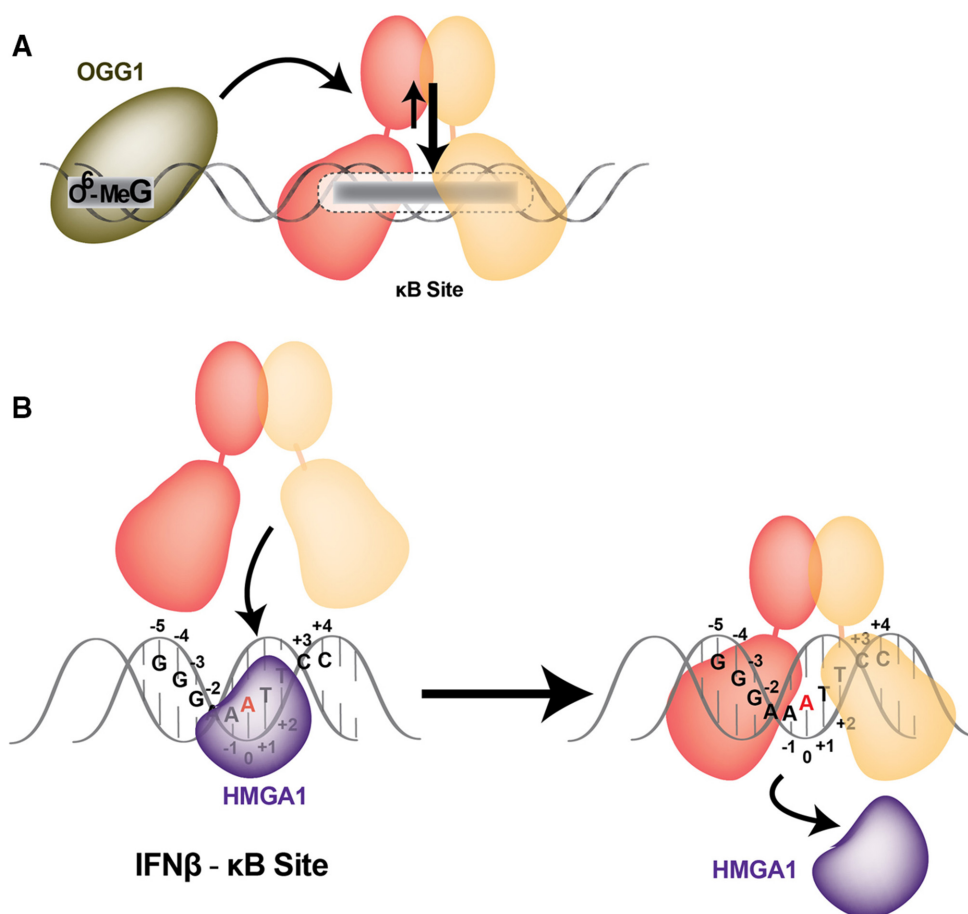


Figure 10. Co-factors affecting NF- κ B:DNA complex altering DNA structural conformation. (A) DNA repair protein OGG1 enhances the recruitment of RelA dimers to the κ B sites through binding to an O^6 -methyl guanine damaged site (O^6 -MeG). Upon OGG1: O^6 -MeG binding, the κ B site structure is altered favoring NF- κ B binding. (B) Architectural transcription factor HMGA1 enhances the recruitment of RelA dimer to the IFN β - κ B site. The proposed model shows how HMGA1 transiently bind the DNA minor groove affecting the conformation of the DNA major groove facilitating NF- κ B binding.

ing the DNA binding properties of RelA. Residues that have been observed to become modified in response to treatment of cells with pro-inflammatory cytokines include Arg30, Lys37, Lys218, Lys221 and Lys310 (130–133) (Figure 11). Both Arg30 and Lys37 are located within loop L1, which has been discussed previously and is critical for mediating base-specific contacts with κ B DNA. Interestingly, neither Arg30 nor Lys37 are directly involved in DNA contacts (132). Although a detailed mechanism that explains how modification of these residues affects DNA binding remains to be deduced, their positions within of loop L1 suggest that the effect is likely indirect through altering the conformation of nearby loop L1 residues that directly contact DNA. In support of this hypothesis, it is worth pointing out that some amino acid residues from the DNA base contacting loop L1 interact with one another to help stabilize the loop L1 conformation and allow it to contact DNA as a stable, prearranged unit. In the NF- κ B p50 subunit, for example, Glu60 bridges Arg54 and Arg56 as together they contact DNA as a structured module. The stability and utility of this folded polypeptide structure were illustrated when it was found to be exploited by RNA in selection binding experiments (134,135). In RelA and c-Rel, a similar Glu brings to-

gether one of the two loop L1 Arg residues and the Arg from the interdomain linker. Residues Lys218 and Lys221 have been identified as sites of acetylation upon RelA activation (130,131). These residues are positioned directly above the central base pairs. Although they do not directly contact DNA, acetylation of these lysine residues could extend them closely enough to influence DNA bending at the center of the complex or even participate in direct contacts with DNA bases that prevent efficient binding. Lys310 is present within the flexible, NLS-containing region at the RHR C-terminal end, which affects subcellular localization of RelA and could influence function of the C-terminal TAD. Therefore, it is expected that post-translational modifications of RelA within the RHR will impact DNA binding affinity and their impact could be DNA sequence dependent.

The most extensively studied RelA post-translational modification is the previously mentioned phosphorylation at Ser276. This residue is targeted for phosphorylation by two different kinases, MSK1/2 and protein kinase A (PKA), and its phosphorylation appears to be essential for RelA transcriptional activity (136–139). Mice expressing a version of RelA in which Ser276 is mutated to Ala demonstrate embryonic lethality, a phenotype that is sim-

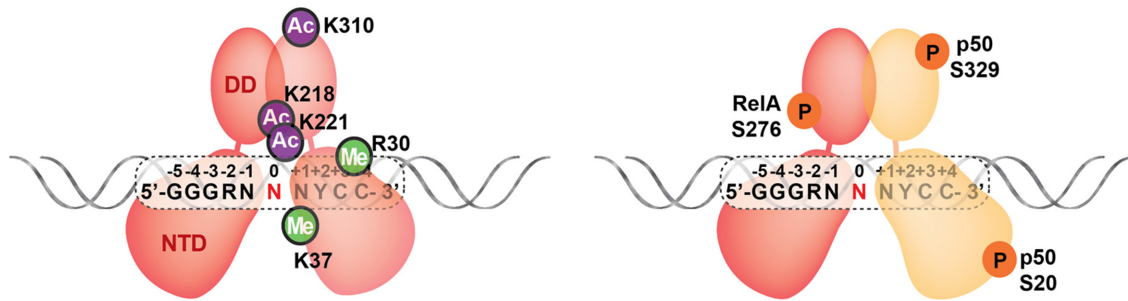


Figure 11. Post-translational modifications affect NF- κ B DNA binding. Acetylation and methylation (left) and phosphorylation (right) of different NF- κ B subunits affecting NF- κ B dependent transcription through altered DNA binding.

ilar to complete knockout of the gene, and exhibit severe developmental defects, most notably in eye development (137). Surprisingly, the Ser276Ala mutant affects expression of only a subset of NF- κ B target genes and the mutant RelA binds DNA with near equal affinity as the wild type protein. This suggests that phosphorylated Ser276 (p-Ser276) provides additional functionality to RelA that is required for expression of a select subset of genes including some that are critical for development. One likely possibility is that p-Ser276 facilitates CBP/p300 recruitment, as discussed previously. Therefore, phosphorylation may play a role in changing chromatin dynamics through acetylation activity of CBP/p300. It deserves mentioning that replacement of Ser276 with a phosphomimetic Glu residue results in a RelA mutant that completely fails to bind DNA *in vitro* (unpublished observation). Together, these results suggest a complicated and incompletely understood role for p-Ser276 in RelA function. One possibility is that only a subset of total cellular RelA undergoes phosphorylation at Ser276 and the two forms of the protein, phosphorylated and unphosphorylated, are involved in two distinct functions. Under this model, the unphosphorylated protein might be directly responsible for transcriptional activation by binding to κ B sites while the phosphorylated form could be involved indirectly in chromatin regulation and/or act as co-factors to regulate binding of other TF to their DNA sites discussed above. In addition to Ser276, RelA is a substrate for phosphorylation at multiple sites within its TAD, but none of these modifications has any impact on DNA binding.

The NF- κ B p50 subunit also undergoes phosphorylation at various locations (140). One of these sites is Ser329 (141). This DNA damage-induced cytotoxic phosphorylation primes the p50:RelA heterodimer to avoid binding κ B sites with C at the -1 position, such as GGGACTYYCC, without affecting binding to GGGAATYYCC type of sequences (-1 bp is underlined). Phosphorylation-dependent κ B site binding selectivity prevents expression of anti-apoptotic factors and tips the balance of cell stress signaling toward cell death. Surprisingly, the phosphorylation site of p50 is located near the tip of the dimerization domain, far from the protein-DNA interface (Figure 11). Cells that express a phosphorylation-resistant version of p50 (Ser329Ala) function properly, as do cells that lack a p50 subunit entirely (141). This suggests that other NF- κ B dimers can substitute the function of p50:RelA heterodimers. It is possible that p-Ser329 allosterically af-

fects DNA binding of p50. Without further structural and biochemical data, it is difficult to predict how structural changes at a distal site propagate to residues that directly contact DNA in a manner that alters binding specificity. It is also possible that a co-factor specific to the p50 subunit is critical for guiding p50 to bind DNA. Phosphorylation at Ser329 could block binding to such a co-factor and lead to reduced DNA binding. Ser337 and Ser20 of p50 have also been shown to undergo phosphorylation (142) (Figure 11). Phosphorylation at Ser20, which is not conserved in murine p50, affects p50:RelA heterodimer binding to VCAM- κ B sites. There are two κ B sites within the VCAM promoter, both of which deviate from the consensus at positions -1 and -2 (143). Ser20 is located within an apparently flexible N-terminal segment, which is not directly involved in DNA contacts. How this phosphorylation might alter DNA binding specificity of p50 requires further study. Ser337 is homologous to Ser276 of RelA and, similar to RelA Ser276, Ser337 of p50 also undergoes phosphorylation. Whereas phosphorylation at this site by protein kinase A (PKA) was shown to augment DNA binding by the p50 homodimer in cells (144), both homodimerization and DNA binding are negatively affected by the phosphomimetic Ser337Asp *in vitro* (145). These observations suggest that p-Ser276 of RelA and p-Ser337 of p50 might be targeted by other factors to enhance DNA binding *in vivo*.

I κ B proteins influence DNA binding

The inhibitor of NF- κ B (I κ B) proteins employ a centrally located ankyrin repeat domain (ARD) to associate noncovalently with select NF- κ B dimers. I κ B proteins are classified into three groups: the classical I κ B group includes I κ B α , I κ B β and I κ B ϵ ; the 'precursor' I κ B proteins p100/I κ B δ and p105/I κ B γ give rise to the mature NF- κ B p50 and p52 subunits, respectively; and the nuclear I κ B group includes Bcl-3, I κ B ζ and I κ BNS (63,146). I κ B binding typically renders NF- κ B incapable of DNA binding and partitions the transcription factor out of the nucleus.

Nuclear I κ B proteins do not act as typical inhibitors that form inhibitory NF- κ B:I κ B complexes in the cytoplasm. Rather, these proteins regulate NF- κ B function in the nucleus by acting as signaling and target gene-specific 'co-activators' of transcription (147) (Figure 12). The expression of all three nuclear I κ B proteins is regulated by NF- κ B, primarily through RelA and c-Rel dimers. Upon trans-

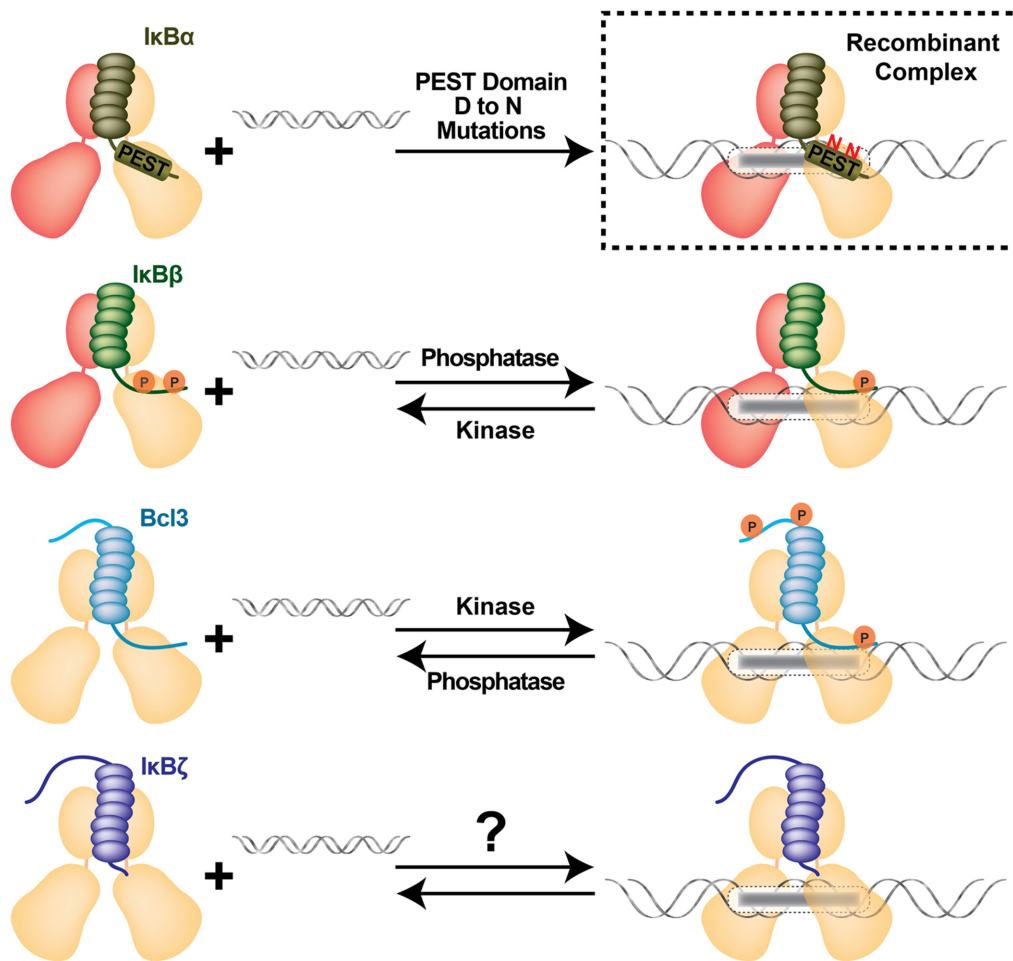


Figure 12. Nuclear I κ B proteins affect transcription by forming NF- κ B:I κ B:DNA complexes. Wild type I κ B α forms stable binary complexes with NF- κ B (top left). Mutations on the acidic I κ B α acidic PEST C-terminal domain partially favor ternary complexes with NF- κ B and DNA. Such ternary complexes have not been seen *in vivo* (boxed). Nuclear I κ B β forms ternary complexes with NF- κ B and DNA only in its hypo-phosphorylated form. These promoter specific complexes induce gene transcription. Bcl-3:NF- κ B:DNA ternary complexes occur only when Bcl-3 is phosphorylated. Unphosphorylated Bcl-3 acts like classical I κ B proteins *in vitro*. It is possible that specific phosphatases exist *in vivo* that convert Bcl-3 into a classical I κ B protein. Finally, I κ B ζ is known as a transcriptional coactivator by forming ternary complexes. It remains to be determined whether it is regulated similarly to the other nuclear I κ B proteins.

lation, nuclear I κ B proteins localize to the nucleus and associate with NF- κ B p50 and p52 homodimers on DNA as ternary complexes. Ternary complex formation by Bcl-3 requires that it first be phosphorylated, as unphosphorylated Bcl-3 favors formation of binary complexes with p50 and p52 (148). However, the phosphorylation pattern of Bcl-3 is highly complex and several Bcl-3 phospho-isoforms coexist in cells, each of which can potentially regulate a distinct subset of genes. Two of the essential phosphorylation sites are located within a structurally flexible region C-terminal to the ARD. Since the C-terminus is not involved in binary complex formation with p50 or p52, it is reasonable to suppose that phosphorylation within this region enables Bcl-3 to make additional contacts with p50/p52 allowing them to associate with DNA (148). (Figure 12). In the absence of structural information, it is unclear why unmodified Bcl-3 cannot form a ternary complex with κ B DNA and NF- κ B and how phosphorylation enables ternary complex formation.

I κ B ζ selectively binds the p50 homodimer and, as part of a ternary complex on DNA, I κ B ζ makes direct specific contact with the promoter DNA. This explains how I κ B ζ :p50 recognizes specific promoters to regulate downstream genes (44,149). Interestingly, although I κ B β does not associate with NF- κ B and DNA forming a ternary complex under normal conditions where it is hyperphosphorylated (pp-I κ B β), a specific partially dephosphorylated state of I κ B β , referred to as its hypo-phosphorylated form (p-I κ B β), can form ternary complexes at specific promoters similar to the Bcl-3:NF- κ B:DNA complexes (Figure 12). Unlike Bcl-3, which associates with p50 or p52 and DNA on a large number of promoters and affects the expression of hundreds of target genes, promoter association of p-I κ B β is highly selective. Only expression of IL-1 β and TNF- α has been shown to be regulated by p-I κ B β (150–153). I κ B α also can bind DNA as a ternary complex with NF- κ B. But this ternary complex is extremely transient and was observed only as a rapid first step in removal of NF- κ B from an ac-

tive promoter to down-regulate gene expression (154,155). Mutations within the C-terminal acidic residues of I κ B α alter its NF- κ B ‘stripping’ function resulting in a more stable ternary complex (156). Thus, it appears as though the gene regulatory activity of I κ B proteins at promoters is a function of the amount of time they typically spend bound to NF- κ B on DNA. If the half-life is very short, the I κ B function as repressors by catalyzing removal of NF- κ B from the promoter DNA. If, on the other hand, I κ B binding extends the amount of time an NF- κ B dimer remains bound to DNA, then the I κ B takes on the function of transcriptional ‘co-activator.’ Modifications, primarily by phosphorylation, enable different I κ B proteins to act as transcriptional regulators of specific genes through promoter recruitment. Interestingly, multiple phosphorylation events on Bcl-3 convert it into a transcriptional co-activator, while hyperphosphorylation renders I κ B α and I κ B β more effective inhibitors of transcription. The simple temporal model presented here suggests that I κ B α and I κ B β can fulfill roles as transcriptional co-activation if negative charges of their C-terminal segments are reduced or shielded (Figure 12).

With regards to the influence on transcriptional regulation, I κ B proteins can be viewed as a special class of co-factors. Unlike the previously described co-factors, which interact with NF- κ B weakly and transiently to facilitate DNA binding, p-Bcl-3, I κ B ζ , and p-I κ B β bind NF- κ B with considerably higher affinity (44). The stable interaction with NF- κ B is essential for Bcl-3 and I κ B ζ since they regulate the transcriptional activity of p50 and p52 homodimers, which lack their own transcriptional activation domains. The more transient interacting protein co-factors, on the other hand, only regulate DNA binding by RelA, c-Rel, and possibly RelB. In addition to stabilizing DNA binding by NF- κ B p50 and p52 homodimers, both Bcl-3 and I κ B ζ regulate selective promoter DNA binding and the events downstream of DNA binding such as histone acetyltransferase and deacetylase recruitment (149,157,158).

Chromatin influences NF- κ B binding to DNA

Unlike the pioneering transcription factors such as FOXA and GATA, among others (159,160), NF- κ B has no inherent ability to free target DNA from its association with chromatin. Indeed, the nucleosome acts as a barrier for NF- κ B to induce gene expression. Consequently, several NF- κ B target genes exhibit delayed expression since their target κ B sites are masked by nucleosomes and they must await remodeling factors to free the sites (161–163). It has been reported that NF- κ B facilitates RNA Pol II elongation and it is known to interact with transcriptional co-activators such as CBP/p300 (164–166). Presumably, the interaction between NF- κ B and co-activators is in synchrony with its DNA binding. Whether NF- κ B simultaneously interacts with a neighboring nucleosome is not known. Over a period of nearly ten years, a series of studies were carried out that seemed to suggest that NF- κ B could bind to κ B sites hidden within the nucleosome DNA *in vitro* (167,168). However these experiments were performed with DNA sequences that were not optimum and failed to form nucleosomes akin to the established 601 nucleosome positioning

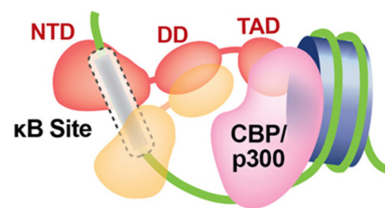


Figure 13. A model of NF- κ B and the transcriptional coactivator CBP/p300 bound to a κ B site in a chromatin context. The NF- κ B RHR binds the κ B site while its TAD contact CBP/p300 and/or nearby nucleosomes simultaneously. Similar ternary complexes with co-repressors and nucleosome could also be possible.

sequence (169). Further analysis revealed that under dilute concentrations the non-ideal nucleosome assemblies could possibly lose H2A and H2B dimers, thus allowing NF- κ B to bind the unstable half-nucleosomes with κ B sites exposed. NF- κ B can, however, competitively displace the linker histone H1 (170).

These experiments seem to suggest that NF- κ B is only capable of binding to DNA sequences that are completely free of nucleosomes or, at most, to half nucleosomes where H2A/H2B dimers are removed (170). However, neither the precise extent to which a κ B site needs to be cleared of histone proteins to enable NF- κ B binding nor the effect of one bound NF- κ B on movement of nearby nucleosomes are well known. These are critical measures as they could provide support for a mechanism for NF- κ B-dependent effects on PolII elongation. We speculate that protein co-factor facilitated DNA binding by NF- κ B is a critical early event that, followed by regulated binding by CPB/p300, nuclear I κ B, or possibly other co-activators, initiates the process of moving histone octamers and clearing more of the promoter. This process then facilitates binding of additional transcription factors to neighboring sites, including additional NF- κ B subunits on promoters with multiple κ B sites or as non-DNA binding co-factors. Interestingly, many NF- κ B target genes contain κ B sites ~12–500 bp from the TSS. This fairly well conserved length of DNA might give some clue as to the distances covered during the steps of proper nucleosome sliding/eviction from the chromatin and subsequent assembly of complexes formed around NF- κ B as well as other factors. Transcriptional co-repressors could function by interacting with the nucleosome to halt its progress or by targeting NF- κ B by competing for co-activator binding. A neighboring nucleosome thus could act as facilitator of cooperative binding between NF- κ B and coactivator/corepressor as predicted previously (Figure 13) (171). The relative frequency of interaction of co-activators or co-repressors with DNA-bound NF- κ B and/or nucleosomes might then dictate the efficiency of mediator/Pol II binding and hence transcriptional output. Interference by classical I κ B proteins that can actively strip NF- κ B from DNA would serve to decrease the half-life of NF- κ B:DNA complexes disrupting the entire process. Under this model, the interplay between factors that support or block NF- κ B binding to DNA combined with additional NF- κ B-dependent factors that control clearance of DNA from nu-

cleosomes might be sufficient to ensure that all subsequent downstream events leading to transcription initiation or repression ensue.

CONCLUDING THOUGHTS

X-ray crystal structures of an array of NF- κ B:DNA complexes detail the stereochemical foundation of the thermodynamically and kinetically stable endpoint of the protein-DNA interaction. Intermediates along the path of recognition and binding, however, are unknown. This is the true for almost every TF:DNA complex studied to date. A vast body of experimental results from genetics, biochemical, and cell-based experiments revealed that numerous factors affect this binding event. As discussed in this report, these regulators of DNA binding can be divided into three categories: (i) the composition and concentrations of ions that can shield the interacting functional groups on key NF- κ B residues from making direct contact with DNA thus acting as a negative regulator; (ii) the DNA sequence itself both within its core recognition part (κ B sites) and flanking segments, which together determine both local and overall geometry and dynamics of the DNA and (iii) co-factor proteins and covalent modifications to NF- κ B, which not only negate the shielding effect of ions but also induce the interacting protein side chains to adopt specific conformations to facilitate DNA contacts. While the impact of the first two regulators can be easily verified by biophysical and biochemical experiments, assessing the relative contributions to DNA binding by NF- κ B modifications and co-factors is considerably more complicated. Although very different in chemistry, both covalent modification, at least in some instances, and co-factors, in all cases, regulate DNA binding through allosteric mechanisms in which through bond and through space effects lead to modulation of the DNA binding surfaces on NF- κ B. The mechanism of co-factor protein facilitation of DNA binding by NF- κ B is at least partially worked out. Diverse co-factor proteins contact NF- κ B directly and specifically around a surface on the NTD that is distal to the DNA binding surface. This interaction is then relayed to the residues that directly contact DNA. In addition to priming DNA binding residues, these allosteric changes may also serve to alter the shielding effect by ions. The combined effects of various extrinsic (other factors) and intrinsic (protein and DNA sequences) factors determine the kinetics of binding, which is converted into transcriptional output.

One challenge with which we are currently faced is to identify new modifications of specific residues on NF- κ B that influence its DNA binding and transcriptional activation functions. Another is to identify new co-factors that regulate DNA binding. Together with these discoveries, efforts are needed to determine mechanistically how these modifications and co-factors regulate NF- κ B binding broadly to κ B DNA in general as well as to specific κ B sites. Finally, in this review, we have discussed DNA binding by NF- κ B under equilibrium conditions, despite the fact that transcriptional regulation is a process that is wholly dependent upon timing. We have briefly touched upon kinetic control by discussing the consequences on transcriptional activity of Bcl-3 and I κ B β in their different phosphoryla-

tion states. We also speculated that the observed one bp change that switches the potential of a κ B DNA from one that activates transcription to one that represses could be due to its effect on NF- κ B binding kinetics. More and better experiments are required that measure and correlate NF- κ B:DNA binding kinetics and transcriptional output under physiological conditions.

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