Complex NF-κB Interactions at the Distal Tumor Necrosis Factor Promoter Region in Human Monocytes*

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We describe a dense cluster of DNA-protein interactions located 600 nucleotides upstream of the transcriptional start site of the human tumor necrosis factor (TNF) gene. This area was identified as being of potential importance for lipopolysaccharide-inducible TNF expression in the human monocyte cell line Mono Mac 6, based on reporter gene analysis of point mutations at a number of nuclear factor κB (NF-κB)-like motifs within the human TNF promoter region. The area contains two NF-κB sites, which are here shown by DNase I and methylation interference footprinting to flank a novel binding site. UV cross-linking studies reveal that the novel site can also bind NF-κB as well as an unknown protein(s) of approximately 40 kDa. We show that these three adjacent κB-binding sites differ markedly in their relative affinities for p50/p50, p65/p65, and p65/p50, yet this 39-nucleotide segment of DNA appears capable of binding up to three NF-κB heterodimers simultaneously. Reporter gene studies indicate that each element of the cluster contributes to lipopolysaccharide-induced transcriptional activation in Mono Mac 6 cells. These findings suggest that NF-κB acts in a complex manner to activate TNF transcription in human monocytes.

A fundamental problem in infectious disease is to understand the mechanisms by which microbial stimuli such as bacterial lipopolysaccharide (LPS) regulate the expression of human inflammatory mediators such as tumor necrosis factor-α (TNF). Production of TNF by macrophages and monocytes occurs early in infection and orchestrates much of the subsequent inflammatory response (1). Because excessive production of TNF is responsible for many of the pathological consequences of severe infection, its regulation is critical. This occurs at both the transcriptional and post-transcriptional levels (2), with transcriptional regulation showing specificity for both stimulus and cell type (3). Reporter gene studies of the human TNF promoter using a range of stimuli and cell types have identified a number of regulatory elements upstream of the transcriptional start site, mostly within the proximal 200 base pairs (4–13). However, we still have a poor understanding of the precise regulatory events that are responsible for the rapid induction of high level TNF expression in human monocytes and macrophages during acute bacterial infection.

Much debate has centered on the role of nuclear factor-κB (NF-κB) in transcriptional activation of the human TNF gene in monocytes. Although there is strong evidence that murine TNF expression is influenced by a number of NF-κB binding sites (14–16), the situation in humans is less clear. Several lines of circumstantial evidence suggest that NF-κB could play an important role. A number of NF-κB-like binding motifs are located between the start of transcription of the TNF gene and the 3’ terminus of the lymphotoxin-α gene: they are here denoted κB1 (~873 to ~864 nt), κB2 (~627 to ~618 nt), κB2a (~598 to ~589 nt), CK-1 (~213 to ~204 nt (17)), and κB3 (~98 to ~89 nt). It is worth noting that site κB2 is 100% conserved between mouse, rabbit, and human TNF promoters (18). Moreover, LPS induces nuclear translocation of NF-κB in human monocytes, and LPS-inducible TNF expression is suppressed by specific inhibitors of NF-κB mobilization (19, 20). However, studies of the role of NF-κB in the transcriptional activation of the human TNF gene have yielded a conflicting picture. Initial gene reporter studies suggested that NF-κB binding in the human TNF promoter region is of little functional importance when tested in the murine macrophage cell line P388D1 (5) or when analyzed in other contexts such as phorbol 12-myristate 13-acetate stimulation of U937 (myeloblastoid) cells (4) and K562 (erythroblastoid) cells (21). However, recent studies indicate that site κB3 contributes to transcriptional activation of the TNF gene by superantigen (6) and cooperates with an adjacent cAMP-responsive element site to provide an enhancement of TNF transcription in LPS-stimulated human monocyte cell line THP-1 (13).

The present investigation focuses on the role of the other NF-κB binding sites, in particular the conserved site κB2 and its adjacent site κB2a. Several studies have shown that NF-κB-specific complexes are formed when site κB2a is tested against nuclear extracts from stimulated monocytes and macrophages (13, 20, 22), but there has been doubt about their functional importance. For example, reporter gene studies in human THP-1 cells concluded that the region containing κB2 and κB2a does not contribute to LPS inducibility, even though NF-κB-specific complexes were formed at the site κB2a with greater affinity than at the apparently functional site κB3 (13). However, a different picture emerged when reporter constructs...
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containing the human TNF promoter region were transiently expressed in the murine macrophage line ANA-1. In this cell line, which resembles mature bone marrow macrophages, LPS inducibility was found to fall 5–10-fold as a result of deleting the region from −656 to −442 nt (22, 23).

These discrepant observations raise the intriguing possibility that the functional activity of the region around κB2 and κB2a has complex determinants that are specific not only for stimulus and cell type but for the stage of cell differentiation as discussed in Ref. 6. Because the primary concern of the present study is the mechanism of LPS-induced TFN expression in human monocytes, here we have used Mono Mac 6, the human cell line whose surface markers and functional properties most closely resemble those of a mature monocyte (24). In this cell line, we find that mutations in sites κB2 and κB2a sites have a more profound effect on LPS-inducible reporter gene expression than mutations at other NF-κB sites in the TNF promoter region. We further show that sites κB2 and κB2a flank a novel binding site, such that this 39-nt segment of DNA is capable of binding up to three NF-κB heterodimers plus an unknown constitutive factor(s). Each element of this cluster appears to contribute to transcriptional activation of the TNF gene.

EXPERIMENTAL PROCEDURES

Plasmids—Human TNF promoter sequence (−1173 to +130 nt) derived from −1173-CAT construct (19) was used to generate the wild type TNF promoter construct wt (−1173–pXP1) in eukaryotic expression vector pXP-1 (ATCC), and corresponding fragments with point mutations at sites κB2, κB2a and κB3 were used to generate the mutant TNF promoter constructs κB2-mt-pXP1, κB2a-mt-pXP1, and κB3-mt-pXP1. A set of site-directed mutations at the κ site, a mutation at site κB1, and 6–10 insertions between the sites κB2 and κB3 were generated by PCR using −1173-CAT construct as a template and oligonucleotides bearing nucleotide substitutions: κ-mt1 (F:agctGGGGGGTAGTGTGAACACCCGGGG; R:agctTTAAGGGGGGGTCCACCTGG); κ-mt2 (F:agctCCGGGGTTGATTGGGCTTAACCCCG; R:agctGGGGGATTCACCCCGGGG); κ-mt3 (F:agctCCGGGGGTTGATTGGGCTTAACCCCG; R:agctGGGGGATTCACCCCGGGG).

Nuclear Extracts and Electrophoretic Mobility Shift Assay—Oligonucleotides (Oligonucleotides were synthesized with 5′biotinylated tail corresponding to amino acids 2–400 of human p50 and amino acids 31–306 of human p65 were cloned at an expression vector pET32a (Novagen) and used to transform BL21(DE3)LysS bacterial strain. Cultures were grown to O.D.600 = 0.6–0.8, induced with 1 mM isopropyl-1-thio-D-galactopyranoside (Sigma), and then grown for an additional 3 h before harvesting. Bacterial pellets were resuspended in SDS buffer (100 mM Tris-Cl, pH 8.0, 1 mM EDTA) and cleared lysate was absorbed to biotin-labeled probe. Indicated gels were quantified using the PhosphorImager (Molecular Dynamics).

UV Cross-linking and Immunoprecipitation—The binding reaction was performed with radiolabeled oligoduplex corresponding to the site κ in which three central dT residues were substituted with bromodeoxyuridine. DNA probes were generated by PCR amplification using biotinylated primer TNF-480 (TTGTAGCTCTGGAGGCTGTTG) along with [γ-32P]ATP-labeled primer TNF-680 (GCATTATGATCTCCGGGTCC) or vice versa. Following gel purification, radiolabeled DNA probe was adsorbed onto magnetic Dynabeads M-280 Streptavidin (Dynal) according to the manufacturer's instructions. 2–4 × 105 cpm of bead-bound DNA probe was incubated with 5–20 μg of nuclear extract in 160 μl of binding buffer at room temperature for 20 min. Following addition of 160 μl of salt mixture (10 mM MgCl2, 5 μM CaCl2) 0.06–0.25 units RNase A (Boehringer Mannheim) was added to the mixture and incubated at room temperature for 30 s. An equal volume of STOP solution was added (0.5 mM NaCl, 40 mM EDTA, 1% SDS), and digested DNA was recovered using the Magnetic Particle Concentrator (Dynal). DNA was washed with 200 μl of solution I (2 M NaCl, 1% SDS, 0.1 M EDTA) followed by solution II (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) twice. The reaction was analyzed on 7% sequencing gel. Where indicated autoradiograms were recorded digitally using the UVP Image Store 5000 system (UVP Life Sciences, Cambridge, UK) and analyzed with NIH Image 1.6 software (public domain).

Expression and Purification of Recombiant Proteins—The protein sequences corresponding to amino acids 2–400 of human p50 and amino acids 230–306 of human p65 were recovered by PCR using the appropriate primers, p50 and p65 cDNAs were cloned into the bacterial expression vector pET32a (Novagen) and used to transform BL21(DE3)LysS bacterial strain. Cultures were grown to O.D.600 = 0.4–0.6, induced with 1 mM isopropyl-1-thio-D-galactopyranoside (Sigma), and then grown for an additional 3 h before harvesting. Bacterial pellets were resuspended in buffer A (50 mM Tris-Cl, pH 8.0, 0.1 mM EDTA, 0.01% Nonidet P-40, 1 mM dithiothreitol, 10% glycerol) containing 70 mM NaCl and lysed by three sonication cycles. Insoluble material was removed by centrifugation, and cleared lysate was absorbed to biotin-labeled oligoduplex corresponding to consensus NF-κB binding sequence bound to streptavidin-agarose (Sigma) for 30–60 min at room temperature. The agarose beads were washed twice with buffer A containing 100 mM NaCl and the bound protein was eluted with buffer A containing 500 mM NaCl. To form p65/p50 heterodimer, cleared lysates containing equal amounts of recombinant p60 and p65 were mixed and denatured by adding urea to 8 M, followed by step dialysis against buffer A containing 70 mM NaCl and 6, 4, 2, 1, 0.5, or 0 mM urea. The heterodimer was purified from total extract as described above. The purity and concentration of recombinant proteins were assessed using SDS-PAGE gel.

Cell Culture, Transfections, and Luciferase Assay—Mono Mac 6 cells were maintained as described previously (24). Transient transfections were performed on Mono Mac 6 cells by the DEAE-dextran method as described previously (15). Cells were plated in fresh medium at 5 × 104/ml 24 h prior to transfection. The concentration of DEAE-dextran (Amersham Pharmacia Biotech) used was 100 μg/ml. After transfection

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cells were incubated for 24 h prior to LPS activation (1 μg/ml) and for a further 6–12 h before harvesting. Luciferase assay was performed using the Luciferase assay system and Turner Luminometer model 20 (Promega) according to the protocol supplied.

RESULTS

Functional Characterization of the NF-κB-like Sites of the Human TNF Promoter Region in Human Monocytes—To examine whether LPS-inducible specific DNA-protein complexes can be formed at NF-κB-like sites within the human TNF promoter region in human monocytes, oligonucleotide duplexes spanning these sites were tested in EMSA with nuclear extracts from unstimulated and LPS-stimulated Mono Mac 6 cells. LPS stimulation resulted in formation of NF-κB/Rel-specific complexes at sites κB1, κB2, κB2a, and κB3, although they differed considerably in intensity (Fig. 1). Because no NF-κB/Rel-specific complexes were formed at site CK-1 (data not shown), this site was excluded from further analysis. The strongest binding was observed at site κB1, which formed two NF-κB-specific complexes corresponding to p50/p50 homodimer (I) and p65/p50 heterodimer (II), respectively, as defined by using NF-κB/Rel-specific antibodies (data not shown). The binding at the sites κB2 and κB2a was about three times weaker, and the complex formed at site κB3 was at the limit of detection (>10 times weaker than that at site κB1).

To examine the role of each site in LPS-inducible transcriptional activation, we generated a set of reporter constructs with 1.2 kilobase pairs of TNF promoter sequence placed in front of a luciferase reporter gene. Point mutations were created at each of the NF-κB binding sites, using specific mutations that disrupted the formation of NF-κB/Rel-specific complexes when tested by EMSA as above (data not shown). When these constructs were expressed in Mono Mac 6 cells, mutation at site κB1 had little effect on LPS-inducible luciferase activity, whereas mutation at site κB2 or site κB2a resulted in a significant decrease of luciferase activity (approximately 50% reduction of the wild type construct), and mutation at site κB3 had a lesser effect (15–20%) (Fig. 2). Thus, despite the fact that κB1 site appears to have a higher affinity to NF-κB/Rel proteins, sites κB2 and κB2a appear to have a greater effect on transcriptional activation of the TNF gene in Mono Mac 6 cells.

Inducible Occupancy of a Cluster of Three Sites in the Distal Part of the Human TNF Promoter Region—To explore DNA-protein interactions around the κB2 and κB2a sites, we performed DNase I footprinting analysis of a segment spanning −680 to −480 nt using nuclear extracts prepared from Mono Mac 6 cells before or after LPS stimulation. Nuclear extracts made after LPS stimulation gave protection from DNase I cleavage at sites κB2 and κB2a and also in the intervening region spanning −616 to −599 nt (Fig. 3A). Nuclear extracts made before LPS stimulation also gave protection in this region when tested at high concentrations. A similar pattern of protection was observed using the noncoding strand (Fig. 3B).

This observation raised the possibility of a novel binding site between κB2 and κB2a, but it was also possible that the area of protection in between κB2 and κB2a might be because of conformational changes brought about by occupancy of the κB2 and κB2a sites. To examine this question, the above experiments were repeated with DNA bearing specific mutations in κB2, κB2a, or the intervening sequence. Each mutation was found to cause a site-specific alteration in the DNase I footprint (Fig. 3C). That is, mutation of site κB2 abolished the protection of site κB2 (but not of κB2a or the intervening sequence), whereas mutation of κB2a abolished the protection of κB2a (but not of κB2 or the intervening sequence), and mutation of the intervening sequence abolished the protection of the intervening sequence (but not of κB2 or κB2a) (Fig. 3D). We therefore proceeded to characterize the putative novel binding site,
Constitutive and Inducible Binding to the Novel Site, ζ—To examine the binding properties of ζ we used an oligonucleotide duplex matching the sequences from 2619 to 2597 nt as a probe for EMSA. Nuclear extracts were obtained from Mono Mac 6 cells that had been stimulated with LPS (Fig. 4A). For comparison, the binding complexes observed with probes specific for sites kB2 and kB2a are shown. Unstimulated nuclear extracts gave a single major complex with the probe for site ζ. After stimulation this constitutive complex increased in intensity, and on close inspection, a separate sharp band appeared at its lower margin. The latter inducible complex was at maximal intensity between 15 and 120 min after stimulation (data not shown). These data raised the question of whether the novel site might bind two different factors, one constitutively present and the other rapidly inducible in Mono Mac 6 cells.

A further EMSA investigated the specificity of these complexes by competition with various unlabeled oligonucleotide duplexes. The constitutive complex was abolished by competition with 100× itself but not by 100× excess of oligoduplexes corresponding to site kB2 or kB2a (data not shown). In contrast the sharp band of the inducible complex appeared to be inhibited by competition with kB2 or kB2a at least as effectively as by itself. Competition with unlabeled oligonucleotide duplex corresponding to an EGR-1 binding site had little effect on constitutive or inducible complex formation. These results suggested that constitutive and inducible factors interacting with site ζ are of different origin and that the latter might be related to the NF-κB/Rel family.

The distinction between constitutive and inducible binding was also illustrated by the different effects of specific alterations to the ζ sequence (Fig. 4B). A substitution of GTGA → ATCC at 2611 to 2608 nt disrupted both constitutive and inducible binding. Interestingly, an ATTT → GATC substitution at −608 to −605 nt had no effect on inducible binding but abolished constitutive binding. Conversely, these data suggest that a CAC → GGA substitution at −604 to −602 nt might reduce constitutive binding less completely than inducible binding.
**Fig. 4.** Nuclear factors binding to site A. A, nuclear extracts from Mono Mac 6 cells before and after 1 h of stimulation with LPS were used in EMSA with probes corresponding to kB2 (lanes 1 and 2), kB2a (lanes 3 and 4), or the intervening sequence (−619 to −597 nt) (lanes 5 and 6). The A probe shows an discrete inducible complex that migrates slightly ahead of the broader constitutive complex (lanes 7 and 8 show longer exposure for lanes 5 and 6). B, effect of specific mutations on protein binding to site A. Wild type (wt) and different mutant probes (mt1, mt2, and mt3) were incubated with nuclear extracts from unstimulated (lanes 1–4) or stimulated cells (lanes 5–8).

**An Inducible Complex Bound to A is NF-κB (p65/p50) Heterodimer**—The rapid kinetics of the inducible complex suggested the involvement of transcription factor(s) that do not need to be synthesized de novo. Because NF-κB provides a classical example of this and because A includes a sequence (GTGATTTCAC) that has two mismatches with the consensus NF-κB site (GGGGpNNpGCCC, where Pu is purine and Py is pyrimidine), we considered the possibility of an inducible complex being formed by NF-κB proteins. Two items of circumstantial evidence are given above. First, the LPS-inducible complex at A had similar mobility to the complexes at kB2 and kB2a, which are known to bind p65/p50 (Fig. 4A). Second, the LPS-inducible complex at A was inhibited by competition with the p65/p50 binding sequences of sites kB2 and kB2a (data not shown). As a more formal test of this question, we performed an EMSA using specific antibodies against two members of the NF-κB/Rel family, p65 and p50. Both antibodies markedly diminished the inducible complex, whereas the antibodies against another member of the family, c-Rel, and against transcription factor EGR-1 had no effect on this complex (Fig. 5). Taken together, these results identify a major inducible complex formed at site A as p65/p50 NF-κB heterodimer.

**p50 and p65 Exhibit Different Binding Patterns across the kB2/A kB2a Cluster**—Because the various members of the NF-κB/Rel family possess different functional properties, we used recombinant forms of p50 and p65 to examine the possibility that each part of the kB2/A kB2a cluster might preferentially recruit a specific type of NF-κB complex. The recombinant p65 was truncated at amino acid 306 to facilitate bacterial expression and is denoted p65Δ; this truncation does not affect the DNA-binding domain.

The results of methylation interference are shown in Fig. 6. It can be seen that p50 homodimers protect site kB2a much more strongly than site kB2, whereas the opposite is true for p65Δ homodimers. Site A is weakly protected by p50 homodimers but poorly protected by p65Δ homodimers. All three sites are protected by p65Δ/p50 heterodimers.

To provide an indication of relative binding affinity, radiolabeled oligoduplexes corresponding to sites kB2, kB2a, or A were titrated against a standard amount of p50/p50, p65Δ/p50, or p65Δ/p50. DNA-protein complexes were resolved by EMSA and quantitated using a PhosphorImager (Fig. 7A). These data confirm that p50 homodimers bind much more strongly to kB2a than to kB2 or A, whereas p65Δ homodimers bind much more strongly to kB2 than to kB2a or A. However the binding of p65Δ/p50 heterodimers showed less variation between the three sites, being strongest for kB2a and weakest for site A.

In view of the similar affinity of p65Δ/p50 for each of the three adjacent binding sites, the question arises whether the heterodimer can bind at all three sites simultaneously. When recombinant p65Δ/p50 heterodimer was incubated with an oligoduplex corresponding to the full kB2/A kB2a cluster, EMSA revealed a ladder of three complexes (Fig. 7B) in contrast to
the single complexes seen for the individual binding sites. This appearance is consistent with the hypothesis that the cluster is capable of binding one, two, or three heterodimers simultaneously.

All Three Binding Sites Contribute to Inducible Gene Expression in Human Monocytes—To examine whether binding of protein complexes at the site $j$ has any functional effect on transcriptional up-regulation of the TNF gene, we included three different variants of the $j$ site whose effects on DNA-protein interactions are illustrated in Fig. 4B, in our set of reporter constructs with 1.2 kilobase pairs of TNF promoter sequence placed in front of a luciferase reporter gene. When expressed in Mono Mac 6 cells variations at the $j$ site resulted in a significant decrease of luciferase activity in the range of 40–55% reduction of the wild type (Fig. 8A). When a construct with the point mutations at the sites $\kappa B2$, $\kappa B2a$, and $\xi$ was expressed in Mono Mac 6 cells, LPS-induced activity of the gene-reporter dropped even further and resulted in about 70% reduction of the wild type (Fig. 8A).

To explore the effect of proximity of the three sites in trans-activation of the TNF gene, we introduced into the full TNF promoter a spacer of 6 or 10 nt between site $j$ and site $\kappa B2a$, thereby creating an additional half-turn or full turn of the DNA helix. Both insertions resulted in decrease of luciferase activity by about 30% (Fig. 8B).

Thus, each element of this cluster participates in transcriptional regulation of the TNF gene in human monocytes. Interference with the binding of NF-$\kappa B$/Rel proteins to each site of interaction in the region results in 30% of the original TNF gene expression. Repositioning of the sites causes modest re-
FIG. 8. Effect of site-directed and phasing mutations at the site $\xi$ on TNF promoter activity in response to LPS. Cluster of elements between $-627$ nt and $-598$ nt is depicted. Results are expressed as percentages of the fold induction of the wild type construct. Number of independent experiments for each mutant is indicated near the corresponding bar. $\alpha$, mutations in corresponding elements are shown. $\xi$-mt1, $\xi$-mt2, $\xi$-mt3, and $\kappa$B2/$\xi$/B2a each gave significantly lower levels of inducibility than wild type ($w_t, p < 0.01$ for each comparison by paired two-tailed $t$ test). $B$, the position of the 6- or 10-nt insertion is marked. Both 6- and 10-nt insertions gave significantly lower inducibility than wild type ($w_t, p < 0.01$, by paired two-tailed $t$ test).

Characterization of the Constitutive Complex at $\xi$—UV cross-linking experiments were carried out to investigate the nature of the factor(s) that bind to $\xi$. Nuclear extracts from unstimulated Mono Mac 6 cells were incubated with a bromodeoxyuridine-substituted oligonucleotide probe, and the complexes were separated by EMSA. After UV cross-linking, the complexes were excised and eluted from the gel. When the purified complexes were analyzed by SDS-PAGE, a major band was seen at $M_r$ $\sim$ 50 kDa (Fig. 9A). Because the oligoduplex itself migrates at about 10 kDa in this gel system, this would suggest that the constitutive binding factor contains a major component of approximately 40 kDa. UV cross-linking experiments with nuclear extracts from stimulated Mono Mac 6 cells showed the same constitutive component plus two additional bands of $\sim$ 75 and $\sim$ 60 kDa consistent with p65 and p50 (Fig. 9A, lane 2, and B, lane 1). To provide further confirmation that the inducible complexes contain p65/p50 heterodimer, the cross-linked products were immunoprecipitated with monospecific antibodies and then analyzed on SDS-PAGE. Anti-p65 antibodies immunoprecipitated a complex that would correspond to a protein of 65 kDa, whereas anti-p50 antibodies immunoprecipitated a complex corresponding to a protein of 50 kDa, and control experiments with anti-C/EBP antibodies or anti-biotin antibodies did not yield an immunoprecipitated band (Fig. 9B, lanes 2–5). Taken together, these results indicate that site $\xi$ is constitutively occupied by a protein(s) of about 40 kDa and that LPS stimulation causes p65/p50 NF-$\kappa$B heterodimer to bind at the same site.

DISCUSSION

The aim of the present study was to examine the role of NF-$\kappa$B in the regulation of TNF transcription in Mono Mac 6, a cell line chosen because of its close similarity to well differentiated human monocytes (24). When the functional effects of four different NF-$\kappa$B-like sites in the TNF promoter region were compared by point mutagenesis and reporter gene expression, two neighboring sites in the distal part of the promoter region were identified as making the greatest contribution to LPS-induced gene expression. We show that in between these two sites, denoted $\kappa$B2 and $\kappa$B2a, there is a 19-nt sequence that binds both constitutive and inducible nuclear factors. This intervening sequence is referred to here as $\xi$, and the cluster as a whole can be summarized as $-627\kappa$B2/$\xi$/B2a $-598$, although the functional boundaries of the novel binding sites within $\xi$ remain uncertain.

The capacity of the $\kappa$B2/$\xi$/B2a cluster to bind NF-$\kappa$B has two interesting features. The first is that three NF-$\kappa$B complexes can bind to such a short segment of DNA. Whether they do so simultaneously is a matter for further investigation, but DNase I footprinting patterns suggest that this might be the case. A further suggestion comes from a comparison of the EMSA behavior of the individual binding sites with that of the full
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κB2/κB2a region. Whereas the individual binding sites only form one complex when incubated with recombinant p65Δ/p50, the full κB2/κB2a region forms three separate complexes that would seem to correspond to the binding of one, two, or three heterodimers (Fig. 7B).

The second potentially important aspect is the wide variation in binding affinity across the cluster and the fact that different members of the NF-κB/Rel family preferentially bind to different parts of the cluster. Site κB2 has relatively high affinity for p65Δ homodimer but relatively weak affinity for p50 homodimer, whereas the opposite is true for site κB2a. Such preferential binding patterns are likely to be explained by minor sequence differences between the sites (28), yet it is intriguing that we find little variation in binding affinity for p65/p50, the canonical form of NF-κB, between all three sites. As shown in Fig. 7A, the difference in p65/p50 binding affinity between site κB2a, which is strongest, and site ξ, which is weakest, is in the region of only 3–4-fold. We do not know whether the polarization of the cluster in respect to p65/p65 and p50/p50 binding affinity is of functional significance. But it has been observed that the acquisition of LPS tolerance in Mono Mac 6 cells is associated with a shift in the predominant form of NF-κB from p65/p50 heterodimer to p50 homodimer, suggesting that the former activates and the latter tends to suppress TNF expression (29). Thus, it is interesting to speculate that one function of the κB2/κB2a cluster within human macrophages might be to switch LPS-induced TNF expression on and off by allowing active interaction between different forms of NF-κB and the elements of the cluster.

Gene reporter expression was approximately halved by mutations that interfered with NF-κB binding to any of the three sites in the κB2/κB2a region. Artificial spacing introduced between the sites also resulted in reduction of gene expression. These data suggest that each element of the cluster contributes to gene transcription and that the structural unity of this region may be important for the maximal levels of expression. In addition, when NF-κB-specific interactions were ablated throughout the κB2/κB2a region, LPS-induced transcriptional activation was reduced by 70%. Although the present study focuses specifically on the question of TNF regulation in human monocytes, it raises the more general question as to whether p65/p50 in isolation can activate TNF transcription factors to be assembled into higher order enhancer complexes (32). It has been recently demonstrated that cooperation of the κB3 site with an adjacent cAMP-responsive element site provides an enhancement of TNF transcription in LPS-stimulated THP-1 cells (13). Our findings support the notion that an assembly of protein complexes at κB2/κB2a is required for efficient induction of TNF transcription by LPS in Mono Mac 6 cells. Further studies are needed to understand the functional significance of specific interactions that may occur in this region between each of the NF-κB binding sites and between NF-κB and other nuclear factors.

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