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Interaction of AP-1 with a Cluster of NF-κB Binding Elements in the Human TNF Promoter Region

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Transcriptional activation of the human TNF gene involves multiple regulatory elements whose functional properties vary between stimuli and cell types. Here we have used a COS-7 expression system to dissect the transactivating potential of NF-κB binding sites in the human TNF promoter region from other regulatory influences. In this model, NF-κB acts largely through a dense cluster of three binding sites located 600 nt upstream of the transcription start site. We show that the transcriptional activity of this complex is highly sensitive to the p65:p50 ratio that is expressed. We demonstrate that the AP-1 complex c-Jun/Fra2 is capable of binding to this region and that this inhibits the transactivating effects of NF-κB. These results are suggestive of a complex regulatory element that mediates fine control rather than acting as a simple on–off switch for TNF gene expression.

Key Words: **cytokines; gene regulation; transcription factors.**

Tumor necrosis factor (TNF) is a potent proinflammatory cytokine that confers both benefits and risks for the host (1). As well as playing a central role in host defense against infection, it is a major factor in the pathogenesis of infectious and inflammatory disease. The need to maintain an appropriate balance between these risks and benefits, in response to a wide range of different infectious and inflammatory stimuli, poses considerable challenges for transcriptional regulation of the TNF gene. This is cell- and stimulus-specific (2) and involves a variety of regulatory elements sited in the 5′ flanking region (3–6), but much has yet to be learned about how these elements, either individually or in combination, exert fine control over TNF gene expression.

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An intriguing question concerns the functional properties of NF-κB sites within the human TNF promoter region. Whereas the murine TNF gene is undoubtedly regulated through NF-κB (7, 8), the several potential NF-κB binding sites that exist within the human TNF promoter region appeared remarkably devoid of transactivating properties when first analyzed in reporter gene models (4). Cell and stimulus specificity may provide part of the explanation, as there is a growing body of evidence that NF-κB activates human TNF transcription in monocytes and macrophages which are largely responsible for the high levels of TNF produced during acute bacterial infection (9–11). However these observations raise the general question of whether NF-κB sites in the human TNF promoter region serve a more complex regulatory role than a simple on–off switch for gene expression.

The human TNF promoter region contains a cluster of three NF-κB binding sites (kB2, ξ and kB2a) within a 39-nt segment located 600 nt upstream of the transcriptional start site. It is one of the most highly conserved noncoding sequences in the TNF gene region. Using reporter constructs in human and murine monocyte-like cell lines, we have previously shown that LPS-induced gene expression is reduced by over 50% if this region is deleted from the human or murine TNF promoter sequence, or if it is mutated to abolish NF-κB binding (10). In the case of the human sequence, wide variation in binding affinity for different NF-κB isoforms is seen for the three different elements of the cluster, and the central element of the cluster (ξ) also binds an unidentified protein of ~40 kDa that is constitutively present in human monocytes (11).

In this study we have attempted to address several key questions that arise from the above observations. Is NF-κB alone sufficient to activate human TNF gene transcription, and to what extent does this depend on the κB2/ξ/κB2a cluster? How is this affected by disrupting NF-κB binding to different elements of the cluster, or by varying the proportion of different NF-κB isoforms? What is the nature of the unknown protein that

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binds to the ξ element, and how does this affect the ability of NF-κB to activate TNF transcription?

MATERIALS AND METHODS

DNA constructs. Human p50- and p65-expressing constructs in Rc/CMV vector (Invitrogen) were previously described (12). The human TNF promoter construct (−1173)-pGL3 and deletion and site-specific mutant constructs were described in (11). The (−83)-pGL3 construct (further referred to as (−83)) was generated by PCR amplification using TNF(−83)-*Bgl*II primer (5′-aatagatctGGAAGTTTTCCGCTGG-3′) and vector-specific primer *Hin*dIII (5′-AATGCCAAGCTTGGAAGAG-3′) and (−1173)-pGL3 construct as DNA template, and subsequently cloned into *Hin*dIII/*Bgl*II sites of modified pGL3-basic vector. The (−83)′ construct was generated by introducing two nucleotide mismatches (T→G at −59 nt and T→C at −55 nt) into the (−83) construct. The region corresponding to κB2/ξ/κB2a cluster was amplified by PCR using the primers: F (*Kpn*I): 5′-aatggtaccTCCGGGGTCAGAATGAAAGA-3′ and R(*Sac*I): 5′-aatagagctcCCTCAGGAAAGGCTGGG-3′ with TNF promoter wild type and site-specific mutant constructs (wt; κB2 mt; κB2a mt; ξ mt; κB2/ξ mt; ξ/κB2a mt, κB2/ξ/κB2a mt) as DNA templates. PCR fragments were cloned into *Kpn*I/*Sac*I sites of the (−83) or (−83)′ constructs.

The protein sequences corresponding to amino acids 1–331 of human c-Jun, amino acids 1–380 of human c-Fos, amino acids 1–272 of human Fra-1 and amino acids 1–327 of human Fra-2 were recovered by PCR using the appropriate primers and total cDNA derived from Mono Mac 6 cells. cDNAs were cloned into the eukaryotic expression vector pcDNA3 (Invitrogen). All constructs were verified by DNA sequencing.

Protein extracts and EMSA. Oligonucleotide probes were radiolabeled with [α-^{32}P]-dCTP (Amersham): HIV-κB: (F: 5′-agctCTACAAGGGACTTTCCGCTGGG; R: 5′-agctCCCA GCGGAAAGTCCCTTGTAG); AP-1: (F: 5′-agctTTCCGGCTGAGTCATCAAGCG; R: 5′-agctCGCTTGATGACTCAGCCGGAA); ξ: (F: 5′-agctCCGGGGGTGATTTCACTCCCCG; R: 5′-agctCGGGGAGTGAAATCACCCCCGG); Mono Mac 6 cells (10–20 × 10^6) were stimulated with 100 ng/ml LPS for 1 h and nuclear extracts were prepared as previously described (13). COS-7 cells were transfected with CMV-p50 and CMV-p65 expressing constructs or CMV-c-Jun, CMV-c-Fos, CMV-Fra1 and CMV-Fra2 expressing constructs and total protein extracts were prepared by lysing cells in the lysis buffer (20 mM Tris–Cl, pH 8.0, 300 mM NaCl, 0.1% NP-40, 10% glycerol) supplemented with protease inhibitors (Boehringer Mannheim). The binding reaction contained 12 mM Hepes, pH 7.8, 80–100 mM KCl, 1 mM EDTA, 1 mM EGTA, 12% glycerol and 0.5 μg of poly(dI–dC) (Pharmacia). Protein extracts (1–4 μg) were mixed in an 8 μl reaction with 0.2–0.5 ng of labeled probe (1–5 × 10^4 cpm) and incubated at RT for 10 min. Where indicated, a competitive cold probe AP-1, CREB (F: 5′-agctGATTGCCTGACGTCAG AGAGC; R: 5′-agctGCTCTCTGACGTCAGGCAATC), AP-2 (F: 5′-agctCTGGGGAGCCTGGGGACTTT; R: 5′-agctAAAGTCCCCAGGCTCCCCAG) or AP-3 (F: 5′-agctTTAGGGTGTGGAAAGTCCCCA; R: 5′-agctTGGGGACTTTCCACACCCTAA) or appropriate antibodies (all from Santa Cruz) were added prior the radiolabeled probe. The reaction was analyzed by electrophoresis in a nondenaturing 5% polyacrylamide gel at 4°C in 0.5× TBE buffer.

UV crosslinking and immunoprecipitation. The binding reaction was performed with radiolabeled oligoduplex corresponding to the site ξ in which three central dT nucleotides were substituted with BrdU. The EMSA gel was UV illuminated at 302 nm for 30 min at 4°C and exposed to autoradiography for 2–4 h at the same temperature. The region corresponding to the DNA-protein complex was excised and the proteins were eluted in 2× SDS buffer (100 mM Tris–Cl, pH 6.8, 200 mM DTT, 4% SDS, 20% glycerol) at 37°C overnight. They were further processed either for SDS–PAGE or for immunoprecipitation as described (14).

Cell culture, transfections, and luciferase assay. Mono Mac 6 cells were maintained as previously described (15). COS-7 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.2 mM L-glutamine and 0.1% glucose. Transient transfections of luciferase gene-reporter and protein expressing were performed on COS-7 cells by using Fugene 6 nonliposomal reagent according to the manufacturer's instruction (Boehringer Mannheim). After transfection cells were incubated for 24 h prior to harvesting. The luciferase assay using a Turner Designs Luminometer Model 20 (Promega) was performed according to the manufacturer's protocol.

RESULTS

NF-κB Has the Potential to Activate Human TNF Gene Transcription

To address the question of whether NF-κB alone is sufficient to activate human TNF transcription, we used a COS-7 expression system to dissect the transactivating potential of NF-κB binding sites in the human TNF promoter region from other regulatory influences. An equimolar mixture of p50- and p65-encoding plasmids (1 μg) was expressed in COS-7 cells together with a reporter construct containing a luciferase gene placed downstream of human TNF promoter sequence extending −1173 nt of the transcriptional start site. Luciferase activity rose 25-fold in the presence of p50 plus p65 compared to cells containing an empty expression vector (Fig. 1). A similar response was observed when the TNF promoter sequence was truncated to −656 nt. However the response was diminished by about 75% when the promoter sequence was truncated to −442 nt or to −284 nt, and by 90% when it was truncated to −83 nt.

These data indicate that NF-κB in isolation is capable of activating human TNF gene expression. Much of the responsiveness to NF-κB appears to localize in the region between −656 and −442 nt (containing a cluster of three NF-κB binding sites denoted κB2/ξ/κB2a) and in the region between −284 and −83 nt (containing the NF-κB binding site denoted κB3).

Functional Activity of the κB2/ξ/κB2a Cluster Depends on Ratio of p50 and p65 Subunits of NF-κB

p50 and p65 readily form both homodimers and heterodimers. Different molar ratios of p50 to p65 plasmids were transfected into COS-7 cells, keeping the total plasmid amount constant at 1 μg. The cellular distribution of NF-κB dimers was analyzed by EMSA with radiolabeled probes corresponding either to consensus κB site from HIV-LTR or to the κB2/ξ/κB2a cluster. As shown in Fig. 2A, approximately equal amounts of p50/p50 and p65/p50 complexes were formed when p50 and p65 expression plasmids were

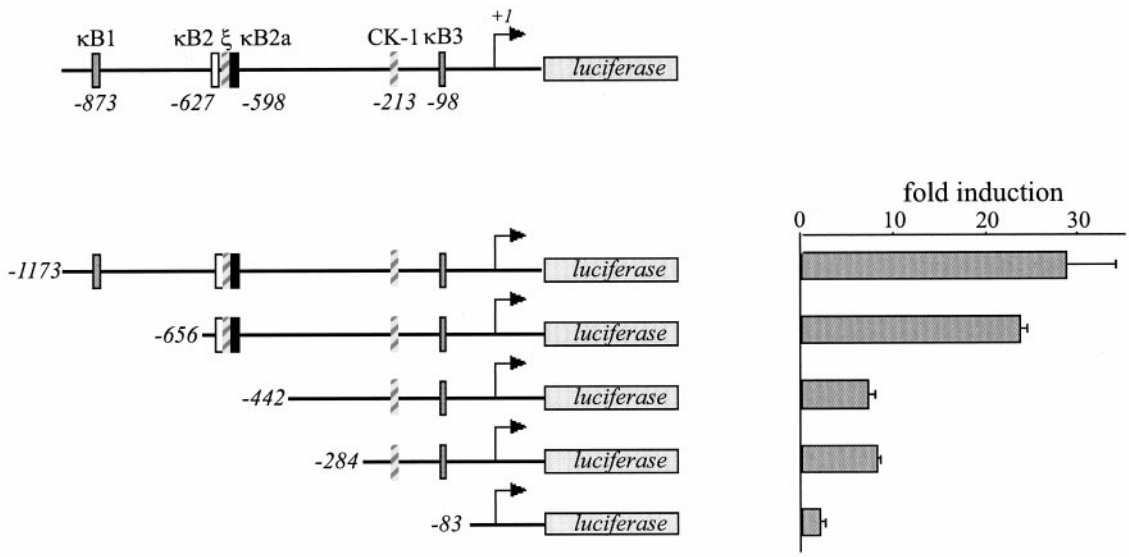

FIG. 1. Effect of 5′ deletions on the TNF promoter activity in response to p65/p50 expression. Luciferase gene-reporter constructs were expressed along with an equimolar mixture of p50 and p65 encoding constructs in COS-7 cells. Results are expressed as mean and standard error of 3 independent experiments.

added in the ratio 1:1, whereas only p65/50 heterodimer was formed when the expression plasmid ratio was 4:1. The identity of the complexes was consistent with previous experiments (11) and was verified by supershift assay (Fig. 2A, right panel).

The same plasmid ratios of p50 to p65 were expressed along with a gene-reporter construct in which κB2/ξ/κB2a cluster was linked to the first 83 nt of the TNF promoter (Cl.wt (−83)). A steady rise in the luciferase activity was observed with increasing amount of p65 expressing plasmid (Fig. 2B) and the activity reached its maximum at the plasmid ratio 4:1 where, according to the above data, p65/p50 heterodimer would be formed exclusively.

We have previously demonstrated that all three elements of the cluster can bind NF-κB heterodimer (11). To determine the contribution of each site to the gene induction by p65/p50 heterodimer, various mutated forms of κB2/ξ/κB2a were compared in 6 independent experiments using a p65:p50 plasmid ratio of 3:1 (Fig. 3, left panel). The site-specific mutations were confirmed by EMSA to disrupt NF-κB binding to sites κB2, ξ or κB2a respectively (data not shown). The following levels of inducibility were observed: wild type > disrupted ξ > disrupted 2 or 2a > double disruption > triple disruption. This result is consistent with previously determined affinities of the sites to p65/p50 (κB2a ≥ κB2 > ξ; Ref. (11)). Each of these differences (i.e., wild type vs disrupted ξ, disrupted ξ vs 2, etc.) was statistically significant ($P < 0.05$ by paired two-tailed t test).

To explore the functional significance of p50/p50 homodimer binding at this cluster we expressed different proportions of p65/p50 and p50/p50 in COS-7 cells ranging between 250 ng and 2 μg of total plasmid (Fig. 2C). A mixture of p65/p50 plus p50/p50 (resulting from a plasmid ratio of 1:1) induced significantly lower reporter gene activity than expression of predominantly p65/p50 (resulting from a plasmid ratio of 3:1), the effect being more prominent at the higher amount of plasmids expressed. This can not be simply explained by the reduction in the total amount of p65, as the reporter gene activity at p65:p50 1:1 plasmid ratio did not follow the linear dose–response curve seen at 3:1 plasmid ratio (Fig. 2C).

Further suggestions of a more complex role of p50/p50 homodimer in this system come from the following experiment. When different mutant constructs were expressed along with the mixture of p65/p50 and p50/p50, in 6 independent experiments (as shown in Fig. 3, right panel), the overall pattern of mutational effects differed from that seen in experiments where p65/p50 predominated. For example, disruption of the κB2, κB2a and ξ sites had similar effect when both p65/p50 and p50/p50 were present, whereas ξ disruption had a much weaker effect than κB2 disruption when p65/p50 predominated.

Taken together, these data indicate that the transactivating effect of the κB2/ξ/κB2a cluster depends on the amounts of both p65/p50 heterodimer and p50/p50 homodimer present in the cell nucleus. When p65/p50 predominates the contribution of each element of the cluster roughly reflects their relative affinities to the heterodimer, but when both p65/p50 and p50/p50 are present, the effect of site-specific mutations is more complex.

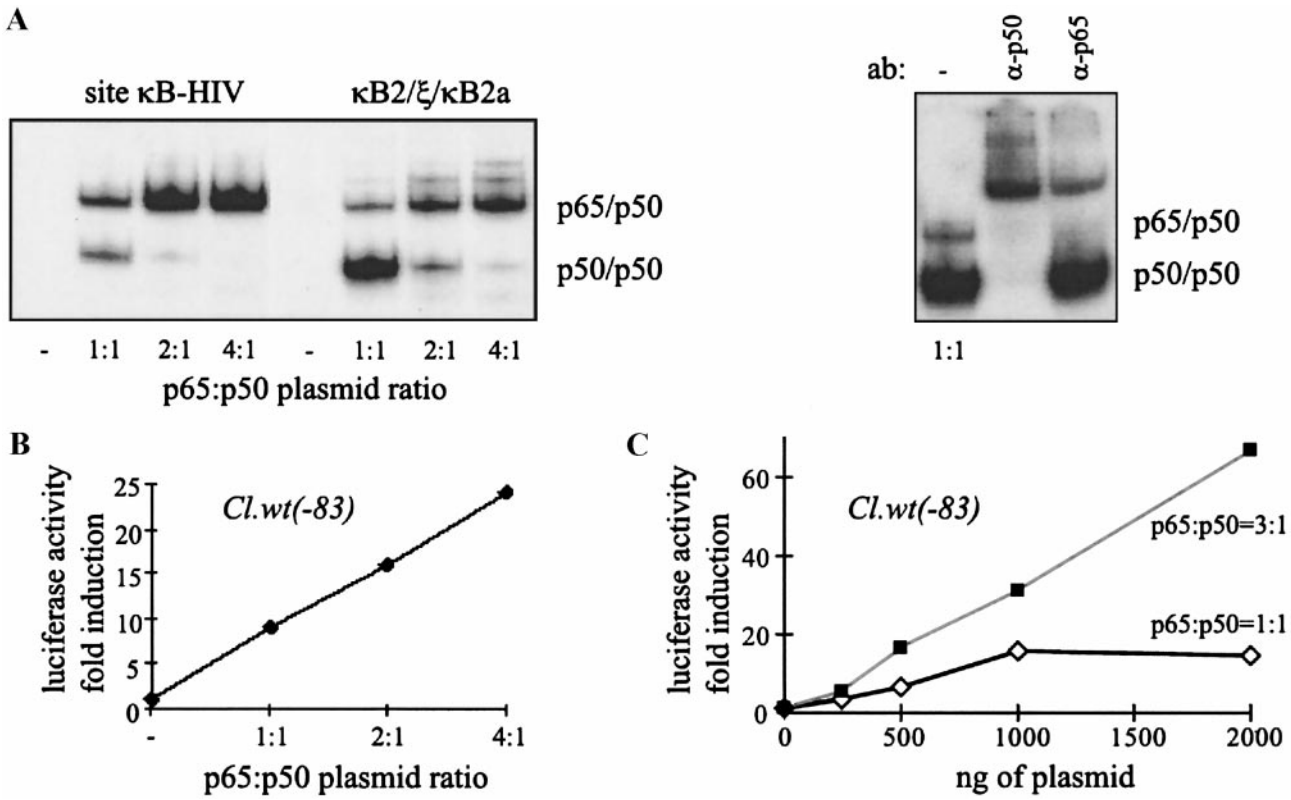

FIG. 2. Comparison of different ratios of p50 to p65 on the κB2/ξ/κB2a cluster activity in COS-7 cells. (A) Total protein extracts from COS-7 cells transfected with different ratios of p50 to p65 expressing plasmids (or an empty RcCMV vector) were used in EMSA with probes corresponding to the consensus κB site from HIV-LTR or to the κB2/ξ/κB2a cluster. The right panel shows supershift assay with anti-p50 and anti-p65 antibodies. (B) Activity of the κB2/ξ/κB2a cluster at the corresponding ratios of p50 to p65. (C) Activity of the κB2/ξ/κB2a cluster at the different amounts of p65/p50 heterodimer (plasmid ratio 3:1) and a mixture of p65/p50 heterodimer and p50/p50 homodimer (plasmid ratio 1:1).

Characterization of Other Factors Interacting with Site ξ

Our previous data demonstrated that site ξ, located in the middle of the cluster, binds NF-κB in LPS-activated monocytes, and an unknown constitutive factor in non-activated (11). Figure 4A demonstrates the relatively diffuse constitutive band together with the sharper band (of similar electrophoretic mobility) that is induced by LPS; only the latter is supershifted by antibodies against NF-κB p50 and NF-κB p65.

We performed series of experiments in non-activated monocytes to identify the nature of the unknown constitutive factor. A panel of consensus sites for known transcription factors (AP-1; AP-2; AP-3; CREB; ISRE; NF-κB; EGR-1; Oct-1; SP1) was used in EMSA competition experiments with nuclear extracts from unstimulated Mono Mac 6 cells. Only two sites, AP-1 and CREB, which differ by one nucleotide, gave clear competition for the binding to the nuclear factors in question. Figure 4B shows an example of a competition experiment for binding to site ξ with itself or with the consensus sites AP-1, AP-2, AP-3 and CREB.

Jun/Fos and ATF/CREB families of transcription factors are able to interact with both AP-1 and CREB site and can form heterodimers with each other. To explore the possibility that the factors interacting with site ξ might be of these families, we tested the effect of antibodies against c-Jun, Fos and ATF-1/CREB proteins on complex formation at the site ξ. As control a consensus AP-1 site was labeled and used together with site ξ in a supershift assay (Fig. 4C). We noted, that the migration patterns of the complex formed at the site ξ and the one at the AP-1 site were similar in this system. The antibodies against c-Jun and ATF1/CREB had little or no effect on the complexes, but anti-Fos antibodies demonstrated a clear complex distortion for both sites. The poor AP-1 complex displacement at the consensus AP-1 site by anti-c-Jun antibodies suggests that these antibodies are not efficient in super-shift experiments.

Further evidence of Fos-proteins being involved in the interaction came from the results of UV crosslinking experiments. When the complex formed in nuclear extracts from nonstimulated Mono Mac 6 cells was

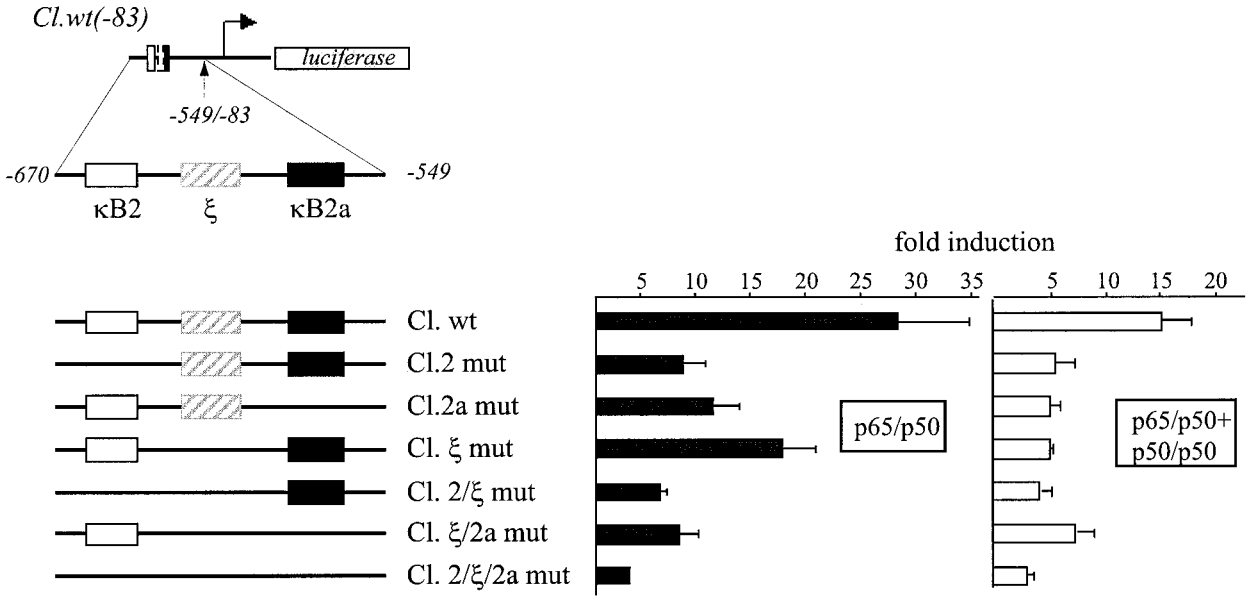

FIG. 3. Effect of site-directed mutations on the κB2/ξ/κB2a cluster activity. Schematic representation of the κB2/ξ/κB2a cluster depicting NF-κB-like sites. This cluster was placed immediately upstream of the first 83 nt of the TNF promoter. Site-directed mutations were shown by EMSA to abolish NF-κB binding to the corresponding site. Mutagenized gene–reporter constructs were expressed together with different p65:p50 plasmid ratios corresponding to predominantly p65/50 (plasmid ratio 3:1, left panel) or to p65/p50 plus p50/p50 (plasmid ratio 1:1, right panel). Results are expressed as mean and standard error of 6 independent experiments.

separated by EMSA, UV-crosslinked and analyzed by SDS–PAGE, two bands were seen at M_r ~50 kDa (strong) and at M_r ~45 kDa (weak) which taking into account the weight of crosslinked DNA corresponded to components of approximately 40 and 35 kDa. The crosslinked products were immunoprecipitated with anti-c-Jun, anti-Fos and anti-ATF1/CREB antibodies and analyzed on SDS–PAGE. Anti-Fos antibodies immunoprecipitated both complexes, while anti-c-Jun antibodies weakly immunoprecipitated a complex of about 50 kDa, and anti-ATF1/CREB antibodies did not yield an immunoprecipitated band (Fig. 4D). UV-crosslinking experiments with nuclear extracts from LPS stimulated Mono Mac 6 cells showed the same constitutive components plus two additional bands of ~75 and ~60 kDa, consistent with p65 and p50 (11). Of interest, the amount of constitutive components appeared to be lower than that before the stimulation.

Taking together these results indicate that the complex constitutively interacting with the site ξ contains Fos-related proteins, and that this may be partially displaced by p65/p50 NF-κB heterodimer when it is induced by LPS stimulation. Based on molecular weight, the Fos-related antigens 1 and 2 are potential candidates.

c-Jun/Fra2 Acts to Suppress Transcriptional Activity of the κB2/ξ/κB2a Cluster

To determine which member of Fos family has the highest binding affinity for site ξ, we expressed c-Fos, Fra1 or Fra2 in COS-7 cells. Based on the fact that Fos proteins do not form homodimers (16) and that anti-Jun antibodies produced a weak signal in the immunoprecipitation, all Fos proteins were expressed together with c-Jun. Protein extracts were incubated with labeled oligoduplexes corresponding to site ξ, a consensus AP-1 site or a consensus NF-κB site, and analyzed by EMSA. As expected, c-Jun/c-Fos, c-Jun/Fra1 and c-Jun/Fra2 complexes were formed at the consensus AP-1 site while no complexes were formed at the consensus NF-κB site (Fig. 5A). The only complex formed at the site ξ was with c-Jun/Fra2, albeit with weaker affinity than to the consensus AP-1 site. Complex composition was confirmed by supershift (Fig. 5A, right panel).

Further experiments were carried out in COS-7 cells to determine the functional effect of c-Jun/Fra2 binding to the κB2/ξ/κB2a cluster. c-Jun plus Fra2 (0.5 μg) coexpressed with a gene–reporter construct containing the κB2/ξ/κB2a cluster linked to the first 83 nt of the TNF promoter (with mismatches at −59 nt and −55 nt to disrupt a potential AP-1 site) −Cl.wt(−83)′ resulted in the same level of luciferase activity as when coexpressed with the (−83)′ construct only (Fig. 5B). However, when luciferase activity of the cluster was induced by expressing p50 plus p65 (0.5 μg) together with the same reporter construct, this was significantly reduced by coexpression of c-Jun plus Fra2 (0.5 μg) (Fig. 5B) but not of c-Jun plus Fra1 (0.5 μg) (data not shown). This result suggests that c-Jun/Fra2 complex

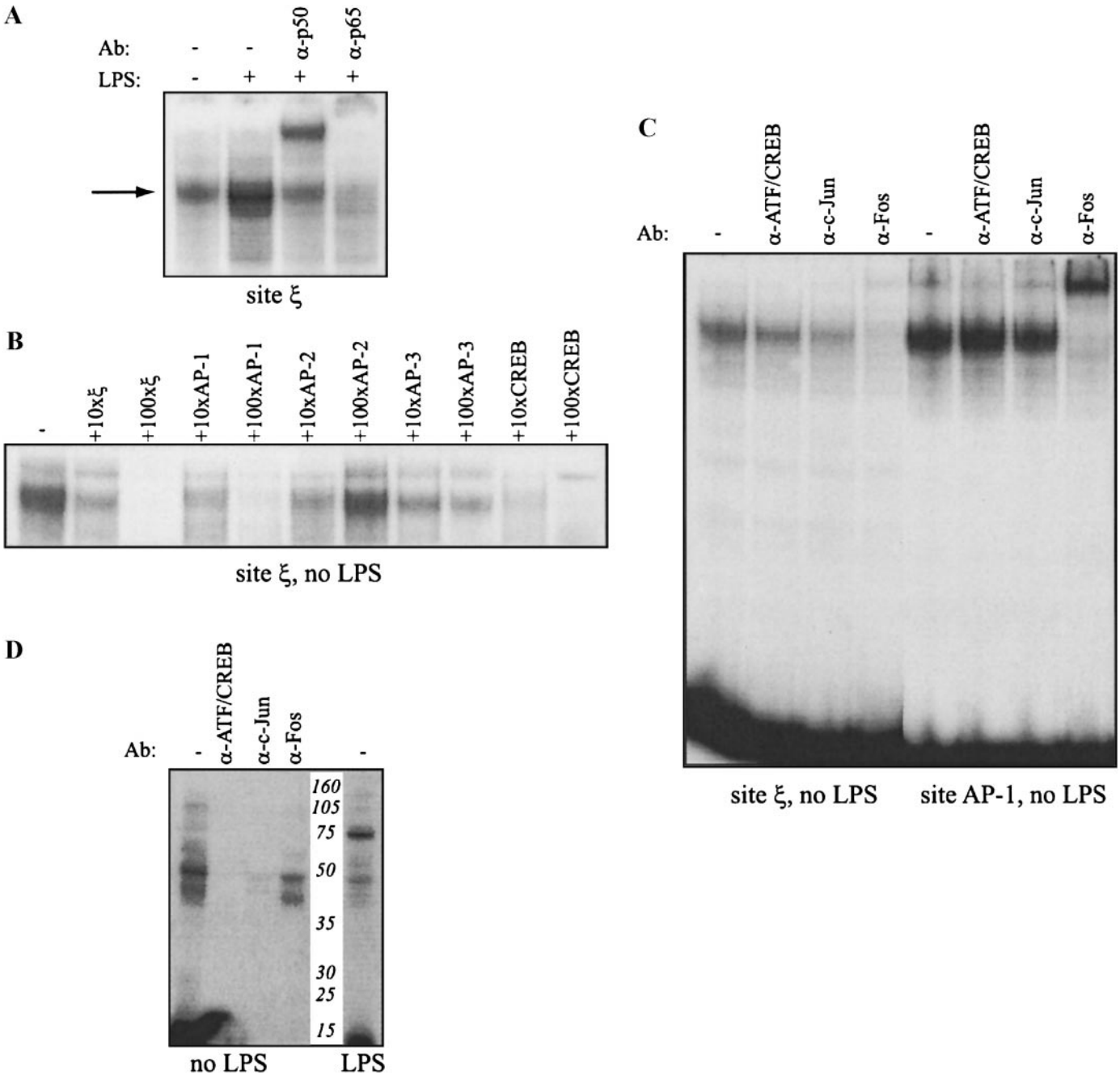

FIG. 4. Different nuclear factors binding to site *ξ*, constitutively and after LPS induction. (A) Nuclear extracts from unstimulated or LPS-stimulated Mono Mac 6 cells were used in EMSA with a probe corresponding to site *ξ*. The LPS-inducible complex consists of NF-κB, as shown by supershift with anti-p50 and anti-p65 antibodies, but the constitutive complex (a more diffused band of higher molecular weight) is not NF-κB. (B) EMSA with unstimulated Mono Mac 6 cells and the probe corresponding to site *ξ*. Where indicated, 10× and 100× excess of unlabeled oligonucleotide duplexes corresponding to *ξ* itself or to consensus AP-1, AP-2 or AP-3 sites was added to the binding reaction. (C) Supershift assay using probes corresponding to site *ξ* or to the consensus AP-1 site. Nuclear extracts from unstimulated MonoMac6 cells in the absence or presence of anti-ATF/CREB, anti-c-Jun or anti-Fos antibodies. (D) Left panel shows UV-crosslinked products of a BrdU-substituted probe corresponding to site *ξ* with nuclear extracts from unstimulated Mono Mac 6 cells (lane 1) and products of immunoprecipitation with anti-ATF/CREB, anti-c-Jun, anti-Fos. Right panel shows UV-crosslinked products using nuclear extracts from LPS-stimulated Mono Mac 6 cells. 10% Tris–glycine–SDS gel with Rainbow full-range molecular weight marker (Amersham).

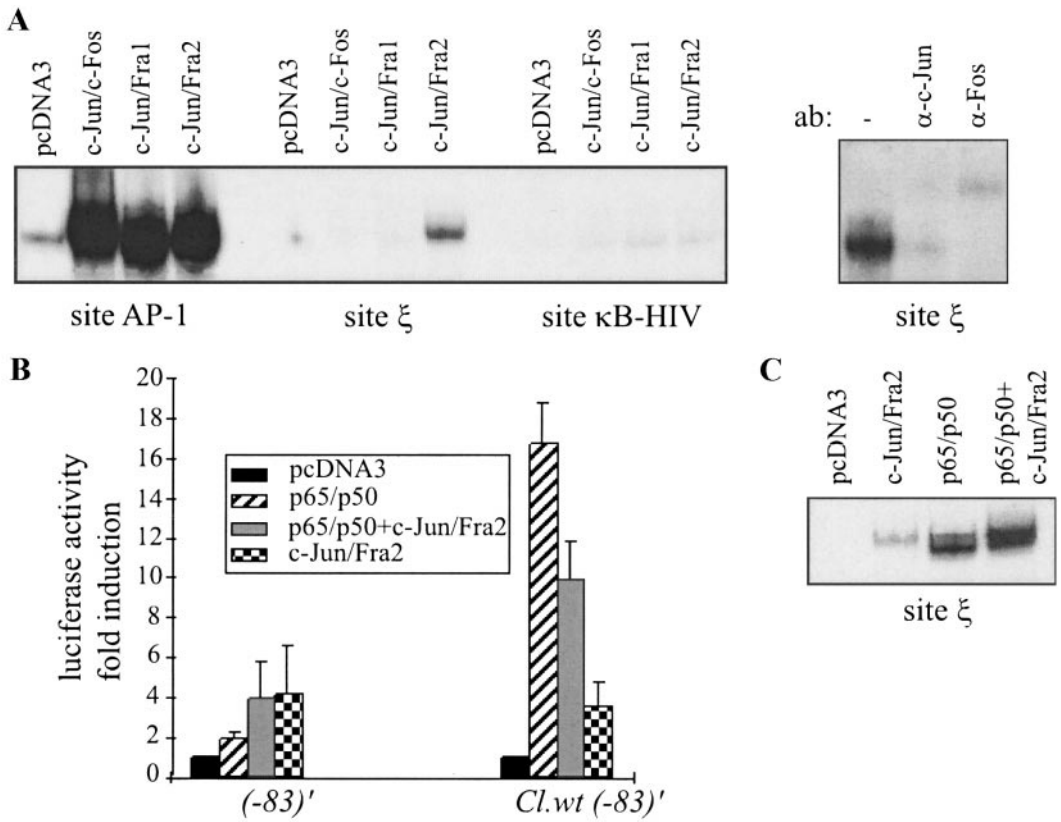

FIG. 5. Binding of AP-1 family proteins to site ξ. (A) Total protein extracts from COS-7 cells transfected either with an empty pcDNA3 vector or with a mixture of c-Jun and c-Fos expressing plasmids; a mixture of c-Jun and Fra1 expressing plasmids; or a mixture of c-Jun and Fra2 expressing plasmids. EMSA with the probes corresponding to the consensus AP-1 site, site ξ or κB site from the HIV-LTR. The right panel shows supershift with anti-c-Jun and anti-Fos antibodies. (B) The Cl.wt(−83)′ and (−83)′ gene–reporter constructs were expressed together with c-Jun/Fra2 or p65/p50 or a mixture of both complexes. Results are expressed as mean and standard error of 5 independent experiments. (C) Total protein extracts from COS-7 cells expressing either an empty pcDNA3 vector or a mixture of p50 and p65 expressing plasmids; a mixture of c-Jun and Fra2-expressing plasmids; or a mixture of p50, p65, c-Jun, and Fra2. EMSA with the probe corresponding to the site ξ.

can specifically inhibit the transactivating effect of NF-κB at this site.

To explore the possibility of c-Jun/Fra2 complex competing for binding to the site ξ with NF-κB complex, we expressed p50, p65, c-Jun and Fra2 in COS-7 cells and analyzed the complex formation by EMSA (Fig. 5C). Both c-Jun/Fra2 and NF-κB complexes were observed, suggesting similar levels of affinity to the site ξ.

Taken together, these results indicate the possibility of complex interplay between NF-κB and c-Jun/Fra2 complexes at the site ξ resulting in reduction of NF-κB transactivating effect on the κB2/ξ/κB2a cluster.

DISCUSSION

A growing body of evidence indicates that NF-κB/Rel transcription factors are necessary for TNF gene activation in monocytes (9–11), but it has been difficult to dissect the transactivating potential of NF-κB binding sites in the human TNF promoter region from other regulatory influences. COS-7 cells expressing exogenous NF-κB provide a useful model system to assess the functional potential of individual κB-binding site in TNF gene transcription. The present findings demonstrate that NF-κB p65/p50 on its own is capable of activating TNF transcription, with a significant part of this effect being exerted through a cluster of binding sites denoted $_{627}$κB2/ξ/κB2a$_{-589}$ that we have previously shown to be involved in TNF transcriptional regulation in human monocytes and murine macrophages (10, 11). Our data indicate that this cluster is an independent enhancer unit which forms complex DNA-protein interactions that may be important for fine tuning of transcriptional regulation.

The different dimeric combinations formed by the NF-κB/Rel family of transcription factors offer the potential for fine-tuning of gene regulation through variation in the relative and absolute abundance of specific dimers. For example, LPS stimulation of human monocytes induces a rapid but relatively transient rise in

nuclear levels of p65/p50 heterodimer followed by a slower and more sustained rise in levels of p50/p50 homodimer (17). In various cell systems, the strong transactivating effects of p65/p50 have been found to be suppressed in the presence of p50/p50 homodimer (18, 19). A natural polymorphism at −863 in the TNF promoter region provided an opportunity to dissect the functional interaction of p65/p50 and p50/p50 at a single NF-κB site (20). This site normally binds both p65/p50 and p50/p50 complexes, but a single base specifically inhibited p50/p50 binding, consequently resulting in higher level of gene expression. Although the mechanism of p50/p50 inhibitory effect is unclear, at least two possible mechanisms have been proposed: competition of p50/p50, which lacks a transactivating domain, for p65/p50 binding sites; or functional interaction of p50/50 with adjacent inhibitory elements (21). Such issues may be relevant to the function of the $_{627}$κB2/ξ/κB2a$_{-589}$ cluster, since we have previously noted that the three component sites differ greatly in their NF-κB binding properties. The site denoted κB2 (−627 to −618 nt) has much higher affinity for p65/p50 heterodimer than for p50/p50 homodimer; the κB2a site (−598 to −589 nt) binds equally well both p65/p50 and p50/p50; and the intervening ξ site (−611 to −602 nt) binds p65/p50 with relatively weak affinity. The experiments reported here demonstrate that the transcriptional activity of the κB2/ξ/κB2a cluster is sensitive to the relative abundance of p65 and p50 proteins in the cell nucleus, with significantly higher levels of activation seen when p65/p50 is the dominant form, compared to when similar amounts of p65/p50 and p50/p50 are present.

These findings raised the question of whether mutations in different parts of the cluster would enhance or diminish transactivation by NF-κB. In this model, the transcriptional activity of the whole cluster was reduced by mutations that disrupted any one of its three elements, and was further diminished by mutations that disrupted more than one element. When all three elements were disrupted, reporter gene expression was reduced by 85%. However the effects of site-specific mutations differed according to the ratio of p65 to p50 that was present. Thus when p65/p50 predominated, disruption of site kB2 caused a more profound reduction of expression than site ξ (70% vs 40%); whereas when similar amounts of p65/p50 and p50/p50 were present, disruption kB2, kB2a or ξ resulted in a similar reduction of around 60%. These data provide further evidence that within this cluster all three binding elements are functionally important; that the activity of the cluster depends on the ratio of p65 to p50 that is present; and that there are complex and poorly understood effects whereby mutations at different points will have different effects in different conditions of stimulation.

A further level of complexity is introduced by the observation that site ξ also appears to interact with unknown factors other than NF-κB (11). The results of competition and supershift EMSA, and of immunoprecipitating UV-crosslinked EMSA complexes, indicate that this site interacts with members of the AP-1 family (22). Direct binding studies with AP-1-like complexes expressed in COS-7 cells indicate that site ξ interacts with c-Jun/Fra2 but not with c-Jun/c-Fos or c-Jun/Fra1. In COS-7 cell co-expression studies we find that c-Jun/Fra2 inhibits the transactivating effects of NF-κB on the κB2/ξ/κB2a. The mechanism of this inhibitory effect is open to speculation, but it is worth noting that site ξ (..GTGATTTCAC..) matches an NF-κB consensus sequence (GGG$^{G}/_{A}$NN$^{C}/_{T}$CCC) at 8/10 positions, and that the two mismatches with NF-κB consensus result in a 6/8 match with a CREB consensus sequence (TGACGTCA). Since the latter sequence is known to have AP-1 binding properties (23), this raises the possibility that c-Jun/Fra2 competitively binds to the same sequence as NF-κB. It may also be relevant that c-Jun/Fra-2 has been previously noted to suppress transactivation by Jun proteins (24), so it is possible than more than one mechanism is involved. It is also interesting to speculate that a binding site that matches the consensus of two different transcription factors can form heterodimeric complexes consisting of both factors. Indeed, the study of hormonal modulation of MHC I gene expression in rat thyroid cells demonstrated the binding of a complex, containing Fra2 and p50 subunit of NF-κB, to an enhancer A (25). We did not find an evidence of p50/Fra2 complex formation in our experimental system of human monocytes, but it is possible that such complex interactions would be dynamic and would depend on other cell-specific factors.

The $_{627}$κB2/ξ/κB2a$_{-589}$ cluster has a number of intriguing features: as well as containing three adjacent NF-κB binding sites, it is one of the most highly conserved noncoding sequences in the TNF gene region (10). The present findings indicate that, when isolated from surrounding regulatory elements, it is capable of activating gene expression. However this process has at least two levels of complexity, namely the preferential recruitment of dimeric forms with different transactivating properties to each of the three NF-κB binding sites within the cluster, and the potential interaction between constitutive AP-1 binding and inducible NF-κB binding in the central part of the cluster. The observation of a constitutive AP-1 complex in monocytes, that appears to be partially substituted by NF-κB following cell activation, suggests that the κB2/ξ/κB2a cluster may participate in suppression of TNF gene expression in resting monocytes and may also be involved in the rapid upregulation of TNF in activated cells. It is likely that similar complexity will be found

in neighboring regulatory elements, and we propose that this may have considerable relevance for stimulus specificity and regulatory fine-tuning of TNF gene expression.

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