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Three NF-κB sites in the IκB-α promoter are required for induction of gene expression by TNFα

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ABSTRACT

NF-κB was first identified as a postive regulator which bound to a 10 bp sequence in the first intron of the Igκ light chain gene. Further characterization of this transcription factor has revealed that NF-κB is kept from binding to its consensus sequence by its inhibitor, IkB-α, which retains NF-κB in the cytoplasm. Upon receiving various extra- and intracellular signals, IκB-α is rapidly degraded and NF-κB is induced to translocate into the nucleus. This process precedes the rapid induction of IκB-α mRNA and protein. To understand how IκB-α is replenished, we have cloned and sequenced the 5′ flanking region of the IκB-α gene and have identified the transcription start site and three NF-κB sites in this region. Further characterization of these NF-κB sites show that they have different affinities for three specific protein complexes which we identify here to consist of various members of the Rel family. In transient assays, cotransfection with a p65 expression vector is able to activate an IκB-α promoter-CAT reporter construct and all three NF-κB sites are required for full activation of the IκB-α gene following stimulation with TNF-α. Our data confirm a transcriptional autoregulatory loop involved in maintaining appropriate NF-κB and IκB-α levels in the cell.

INTRODUCTION

NF-κB and its cytoplasmic inhibitor, IκB-α, belong to the Rel/ankyrin family of transcription factors. The p65 (Rel A) and p50 (NF-κB1) subunits of NF-κkB and other members of this family including p52 (lyt-10, NF-κB2) and the drosophila maternal morphogen, dorsal, share homology with the DNA binding and dimerization domains of the product of the proto-oncogene, c-rel (for review, see references 1, 2, and references therein). Members of this family have been shown to homodimerize and heterodimerize as well (3–7). This interaction between members of this transcription factor family enables them to regulate a wide variety of genes. Such genes include ones involved in, but not limited to, the immune and inflammatory responses, cellular adhesion, and cell growth. NF-κB target sequences have also been found in the enhancer regions of several viruses implicating a role of NF-κB in viral pathogenesis (8–10).IκB-α and other cytoplasmic inhibitors of this family including the chicken homologue, pp40, and the drosophila homologue, cactus, share highly conserved ankyrin repeats which are thought to play a role in cytoplasmic compartmentalization (reviewed in 1 and 11).

IκB-α noncovalently interacts (12) with the NLS (nuclear localization sequence) of both the p50 and p65 subunits that make up NF-κB (13). Thus, IκB-α controls the DNA binding activity of NF-κB by retaining it in the cytoplasm until it is induced to translocate into the nucleus. When the cell receives signals from various sources such as cytokines,viruses, UV light, and others (reviewed in reference 2), NF-κB is released from IκB-α through an as yet unknown mechanism. IκB-α is rapidly degraded, and NF-κB translocates into the nucleus where it binds to its target sequence and regulates the expression of various target genes (11).

Recently, several groups have suggested that IκB-α and NF-κB are involved in an autoregulatory pathway (14–17). These groups demonstrated that induced IκB-α mRNA expression is contingent upon a loss of IκB-α protein and subsequent activation of NF-κB binding activity. To better understand the regulation of IκB-α by NF-κB, we cloned, sequenced, and analyzed the human IκB-α promoter. Here we characterize three NF-κB binding sites in the IκB-α promoter. We show that i) these sites have different affinities for different complexes of Rel family members, ii) in transient transfection assays p65 activates the IκB-α gene and that IκB-α is able to block this activation and iii) all three NF-κB sites are required for full TNFα -mediated regulation of this gene.

MATERIALS AND METHODS

Isolation and sequencing of the 5′ flanking region of the IκB-α genomic clone

An Eco RI/PstI fragment containing the 5′ end of the IκB-α cDNA was used to screen an EMBL-3 human placenta genomic library (a generous gift from C. Rosen) using the techniques

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described by Benton and Davis (18). Two genomic clones were isolated after screening approximately 1×10^6 phage plaques. A 3kb SacI fragment of one of the genomic clones was identified by Southern analysis (19) to contain the 5'end of the IκB-α cDNA and 5' flanking sequences. This 3kb SacI fragment was subcloned into pUC19 and sequenced in both directions by the Sanger dideoxy method (20).

Primer extension analysis

A 19-mer oligo, 400R, was designed in the anti-sense orientation corresponding to nucleotides +83 to +101 in the published IκB-α cDNA (21). This primer was annealed to poly-A+ mRNA harvested from HeLa cells stimulated with TNFα (10ng/ml, Genentech) for 30 minutes. Reverse transcriptase was used to extend the cDNA product using the conditions and protocols previously described (22).

Preparation of nuclear extracts

Nuclear extracts from HeLa cells stimulated with TNFa were prepared as previously described (17). Briefly, HeLa cells were maintained in Eagle's MEM with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were stimulated with TNFα (10 ng/ml) for 15−30 minutes. Cells were then washed twice with cold PBS, transferred into a 1.5 ml eppendorf tube, and spun for 5 minutes at 3500 rpm at 4°C. The cell pellet was resuspended in CE buffer (10mM Tris, pH 8.0, 60mM KCl, 1mM EDTA, 0.3% NP40, 1mM DTT, and 1mM PMSF). Lysed cells were spun for 5 minutes at 3500 rpm. The supernatant was removed and the nuclear pellet was washed in 1ml of CE buffer without NP40 then spun for 5 minutes at 2,500 rpm. The nuclei were resuspended in an equal volume of nuclear extract buffer (20mM Tris pH 8.0, 1.5mM $MgCl_2$, 0.2mM EDTA, 25% glycerol, and 0.5mM PMSF). 5M NaCl was added upon vortexing to a final concentration of 420mM. Nuclei were left on the Nutator at 4°C for 20 minutes. After a spin at 12,000 rpm for 15 minutes at 4°C, the nuclear extracts were collected.

Electromobility shift assays (EMSA)

The binding sites for the three NF-κB sites were created by annealing the oligonucleotides 5'-TCGACCCCAGAGAAATC-CCCAGCCG-3' and 5'-TCGACGGCTGGGGATTTCTCTGG-GG-3' for NF-κB site 1, 5'-TCGACGCTTGGAAATTCCC-CGACCTG-3' and 5'-TCGACAGGTCGGGGAATTTCCAA-GCG-3' for NF-κB site 2, and 5'-TCGACTCCCTGGGGTTT-CCCACGATG-3' and 5'-TCGACATCGTGGGAAACCCC-AGGGAG-3' for NF-κB site 3. 2,000 cpm of the ^{32}P end labeled binding site oligonucleotide was incubated with 2μl of nuclear extracts, 50mM NaCl, 10 mM Tris pH 7.7, 0.5mM EDTA, 1mM DTT, and 2μg poly(dI-dC), and 10% glycerol before loading on a 0.25× Tris-borate EDTA native gel.

For the supershift assays, antibodies to p50, p65, or c-rel (Rockland, Inc., Boyertownm, PA) were pre-incubated with the nuclear extract in the reaction conditions mentioned above before the addition of the labeled probe. Blocking peptides for p50, p65, and c-rel were included in the reaction where described.

PCR mutagenesis of the NF-κB sites

Oligonucleotides containing mutations in each of the three NF-κB sites were used as primers in PCR reactions to generate site specific mutants as previously described (23). The oligonucleotides used to create a mutant NF-κB site 1 are 5'-C-CCCAGAtctATgCtCAGCCAG-3' and 5'-CTGGCTGaGcATa-gaTCTGGGG-3', a mutant NF-κB site 2 are 5'-GCTTGcAA-ATTggCCGAGCC-3' and 5'-GGCTCGGccAATTTgCAAG-C-3', and a mutant NF-κB site 3 are 5'-GacAcAggaaAGGGG-AAAGAAGGCTCA-3' and 5'-ttccTgTgtCACGATCGATT-TGAATT-3' (mutations in the NF-κB sites are in lower case type). The 5' primer, 5'-CCCAAGCTTGCAGCCGACGACC-CC-3', contains a HindIII site and the 3' primer, 5'-GGGGTC-TAGAGGGCGCTGCTGCGGGT-3', contains a XbaI site. These sites were used to clone the wildtype and mutant PCR products into pBL-CAT (24). These constructs were then sequenced using the Sanger dideoxy method (20) to confirm their site specific mutations. These constructs encompass regions −342 to +115 of the IκB-α gene.

Transient transfection reactions and quantitation of CAT activity

Hela cells were cultured in Eagles MEM media supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). The calcium phosphate method was used to transfect the HeLa cells with 10μg of the IκB-α promoter-CAT construct plus pCMV4T-p65 (0.5μg), pCMV4T alone (2.5μg), or pCMV4T-p65 (0.5μg) and pIκB-α (2μg). The total amount of DNA transfected was held constant to 12.5 μg with the pCMV4T empty vector. IκB-α promoter-CAT construct contains the −342 to +115 region of the IκB-α gene cloned into the HindIII/XbaI site of pBL-CAT. The pCMV4T, pCMV4T-p65, and pIκB-α expression vectors have been previously described (25,13). CAT activity was determined by a previously described method (26) and standardized for protein levels (27).

The K562 cells were cultured in DMEM supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). 10μg of wildtype or each of the mutant constructs generated by PCR mutagenesis described above were electroporated into 2×10^6 K562 cells using the BioRad gene pulser at a setting of 300V, 960μF. 24 hours after transfection, TNFa (10 ng/ml, Genetech) was added to the cells. 24 hours following TNFα stimulation, the cells were harvested, rinsed in PBS, and cell extracts were obtained by freezing and thawing the cells three times for 5 minutes in a dry ice−ethanol bath. The cell extracts were incubated with ^{14}C chloramphenicol (Amersham) and Acetyl CoA (Boehringher Mannheim) at 37°C overnight. Acetylated and non-acetylated forms of chloramphenicol were separated by thin layer chromotography. CAT activity was determined by autoradiography and analyzed by densitometry.

RESULTS

Cloning of the human IκB-α promoter

A human placenta genomic DNA library was screened using a 5' EcoRI/PstI fragment from the MAD-3/IκB-α cDNA (21). Two genomic clones were isolated and one of them (clone λ-7), containing a 3kb SacI fragment encoding the 5'end of the cDNA, was further characterized. Sequence analysis showed that the potential 5' flanking sequence contained several putative transcription factor binding sites including ones for Sp1 and NF-κB (Fig.1).

To identify the transcription start site, primer extension analysis was performed. A sequence at position +83 to +101 in the cDNA were used to design an anti-sense oligo, 400R, which was used as a primer to extend poly-A+ mRNA from TNFa stimulated HeLa cells . The primer extension product ends just

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-1275  GAGCTCTGACCGAAGTTTGCTTTATTTTGCCTGTACCAATTCTACCAAG
-1226 TAGATGAATTCAGTCCATGGCTTGCAGGCTTGGAGGTGGAGGTGGAGTGG
-1176 AGAGCATAAATTATATTTCTGCAGGTGAAACCAGAGCATAGTGAGCCGAA
-1126 ACCCCTTTCTACCCAGGTGGCGTGAATGGGTCCAACTGCTACTGTCCCCC
-1076 AGGACAAGAGAGCAAGGAGGGGCGGGCAGGATGGGACTACCTTGAGCCTC
-1026 CCGCAGGATGCCTGGCAACTACTTTCTTAGTGGTCCTTAAGGTCCAATCG
 -976 CGGGTTAAGGCACTAACTAAACTCATTCTTCAGCCTCCTTCTCTAAGTTC
 -926 TCCTCGAATTTGGGTGCCCAGAAGTAGGCTCACGATCCTTTTTCTGCGGG
 -876 AGCACAATGTAGGTCAGATAGCATAAACGAATAGCTACTTATGAACACAA
--826 TAGCTACTCTGCTATTGCAAGGTATCCACAACCACCACTACAAACATTAA
 -776 CACCTTGCTTTACTTGGGAAACAAAAAAAATCATGGTCCTATTCAGCAGT
 -726 TTCCCCATACAGGGAGCGTTTGCCCCTCCCCCAGTCAACAGGGCTGTTCA
 -676 TCCCTAGGAAGTGATTTGAGAGTTCTCCAAGGATTTAGGCTTTCACTCCT
 -626 CCAAAGCTTTCACAACTTCTACCTGGCGGGGGTGCGTGGGGGGGTGGGGG
 -576 CGAACGTAAAAGTTCCTTTGCTGCAAAGAGCCTGGTATAGGCAGAAACAC
 -526 CGGCGCGGCCTGCAGCCCCCTAACCACAGTGCGTCCTTCCCCTTAGAAGT
 -476 CTGGGGAAAGCAAATCCCTACGCCCAGCCATCATTTCCACTCTTGCGTTT
 -426 TCAAAAGATCAAAAACGGAAAGGACCGGCAGTTGGCAAACCCCAAAGAGG
 -376 GACCGCCCATCAGGTCGGCGTCCTTGGGATCTCAGCAGCCGACGACCCCA
                      NFκB site 3
 -326 ATTCAAATCGATCGTGGGAAACCCCAGGGAAAGAAGGCTCACTTGCAGAG

 -276 GGACAGGATTACAGGGTGCAGGCTGCAGGGAAGTACCGGGGGGAGGGGGC

 -226 CTGGTCGGAAGGACTTTCCAGCCACTCGGCGCTCATCAAAAAGTTCCCTG
                                     NFκB site 2A
 -176 TCCGTGACCCTAGTGGCTCATCGCAGGGAGTTTCTCCGATGAACCCCAGC

 -126 TCAGGGTTTAGGCTTCTTTTTCCCCCTAGCAGAGGACGAAGCCAGTTCTC
                                NFκB site 2
  -76 TTTTTCTGGTCTGACTGGCTTGGAAATTCCCCGAGCCTGACCCCGCCCCA
      NFκB site 1
  -26 GAGAAATCCCCAGCCAGCGTTTATAGGGCGCCGCGGCGGCGCTGCAGAGC

  +25 CCACAGCAGTCCGTGCCGCCGTCCCGCCCGCCAGCGCCCCAGCGAGGAAG

  +75 CAGCGCGCAGCCCGCGGCCCAGCGCACCCGCAGCAGCGCCCGCAGCTCGT

 +125 CCGCGCCATGTTCCAGGCGGCCGAGCGCCCCC...
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Figure 1. Nucleotide sequence of the 5′ flanking region of the IκB-α gene. The putative TATA box is in bold type next to the putative transcription start site indicated by the arrow. The three NF-κB sites 1, 2, and 3 are circled and another one, 2A, is labeled. A Sp1 site between NF-κB sites 1 and 2 is double underlined. The translation start site is also highlighted at +132.

adjacent to a TATA-like box (Fig. 2). To confirm this transcription start site and to rule out the possibility that an intron may exist between the 400R oligo and the true transcription start site, RNAse protection assays were done. The size of the protected fragmet coincided with the start site identified in the primer extension assay (data not shown). Thus, transcription appears to begin 36 bases beyond the published cDNA sequence (21) and close to a TATA-like element.

The NF-κB binding sites in the IκB-α promoter bind different members of the Rel family

Computer analysis of the IκB-α promoter originally identified four potential NF-κB binding sites, 1, 2, 2A, and 3 (see Fig. 1). EMSAs (Electrophoretic Mobility Shift Assays) were performed using oligonucleotides to these sites and nuclear extracts from TNFα stimulated HeLa cells. These NF-κB binding sites are not identical in sequence, as shown in Figure 1, and do not bind to identical protein complexes (Fig. 3). The NF-κB site 1 forms a faster migrating minor complex, I, and a slower migrating major complex, II. NF-κB sites 2 and 3 have similar affinities for the proteins which bind to the NF-κB site 1 and these probes bind to an additional protein factor, III, which migrates even slower than the ones mentioned in the above text. The fourth putative NF-κB site, 2A, did not consistently bind to any protein complexes (data not shown).

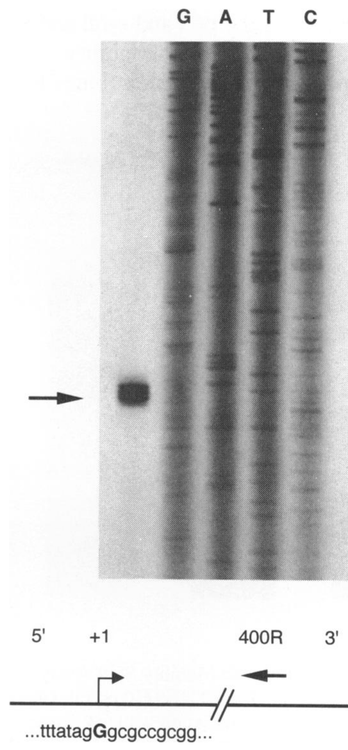

Figure 2. Identification of the transcription start site using primer extension analysis. The primer extension product was run on a 5% sequencing gel alongside a dideoxy sequencing reaction of the IκB-α genomic clone using the same primer that was used in the primer extension reaction. Shown below is a schematic diagram of the primer used in both reactions and the mapping of the transcription start site.

In an attempt to identify the proteins bound to NF-κB sites 1, 2, and 3, antibodies to c-rel and the p50 and p65 subunits of NF-κB were pre-incubated with the nuclear extracts from HeLa cells stimulated with TNFα prior to electrophoresis. Both protein complexes which bind to NF-κB site 1 include p50 (Fig. 4). The antibody to p50 is able to shift the faint faster migrating complex I as well as the darker slower migrating complex II (Fig. 4, lane 3). Incubating the p50 antibody with its blocking peptide is able to impair its ability to supershift both bands (lane 4). Antibodies to p65 or c-rel do not affect complex I but shift complex II (lanes 1, 2, 5, 6). Since the c-rel antibody weakly shifts complex II, the data suggest that complex II is predominantly composed of p50 complexed to p65 and that this complex also contains trace p50/c-rel or p65/c-rel heterodimers. These data also suggest that complex I is primarily comprised of p50 proteins, presumably p50/p50 homodimers, although the possibility of p50/p52 heterodimers has not been ruled out.

Protein complex I described above also binds to NF-κB sites 2 and 3 and is supershifted by the p50 antibody (Fig. 4, lanes 9 and 15) but not the p65 and c-rel antibodies (lanes 7, 11, 13, and 17). Complex II is also able to bind NF-κB sites 2 and 3, and consistent with the data mentioned above, this complex is recognized by antibodies to p50, p65, and c-rel (lanes 7–18) . These observations provide more evidence that complex I consists predominantly of p50 protein and that complex II is a mixture of p50 complexed with either p65 or c-rel.

Complex III, however, is observed with NF-κB sites 2 and 3, but not with NF-κB site 1. This complex is recognized by the antibodies to p65 and c-rel (lanes 7, 11 13, and 17). When

the blocking peptides for the p65 and c-rel antibodies are included in the reaction, they hinder the ability of their respective antibodies to supershift this complex (lanes 8, 12, 14, and 18).

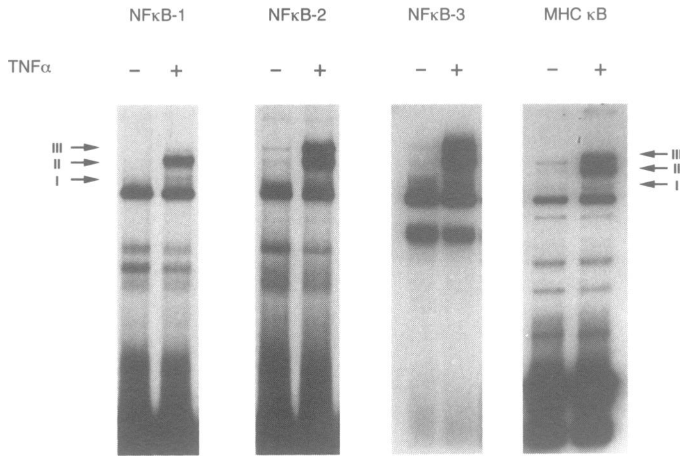

Figure 3. EMSAs (Electophoretic Mobility Shift Assay) of the putative NF-κB binding sites. Nuclear extracts from TNFα (10 ng/ml, Genentech) stimulated and nonstimulated HeLa cells were incubated with ^{32}P labeled oligos made to each of the three putative NF-κB binding sites. The protein/DNA complexes were analyzed on a 0.25× Tris–borate EDTA native gels. The arrows indicate protein complexes I and II bind to all of these sites, while complex III only binds to NF-κB sites 2 and 3. The MHC class I NF-κB site was used as a control.

This complex may also contain p50 since the antibody to p50 also shifts this complex, albeit weakly (lanes 9, 10, 15, and 16). Therefore, although p50 may bind to this site, complex III mainly contains p65 and c-rel proteins which may be present as homodimers or heterodimers.

NF-κB activates IκB-α gene expression

To further characterize the NF-κB sites in the IκB-α promoter, the 5′ flanking region (to position −342bp) was cloned into a CAT reporter construct. Since the p65 subunit of NF-κB has been shown to be a potent transcriptional activator, the IκB-α promoter-CAT construct was transiently transfected into HeLa cells along with a p65 expression plasmid. The p65 subunit of NF-κB was able to stimulate the expression of the CAT gene by 10 fold over transfection of the IκB-α promoter-CAT construct plus the empty pCMV-4T vector (Fig. 5). To further demonstrate that NF-κB is involved in the regulation of the IκB-α gene, the promoter construct was cotransfected together with an IκB-α expression plasmid in addition to a p65 expression plasmid. As expected, IκB-α expression was able to inhibit the expression of its own promoter and CAT activity was inhibited to baseline levels of the IκB-α promoter CAT construct plus pCMV-4T. These data support the idea that NF-κB and IκB-α are involved in an autoregulatory loop.

To determine whether all three NF-κB sites are required for the regulation of the IκB-α gene, we used PCR site directed mutagenesis to alter the NF-κB sites at the residues which have been shown to interact with NF-κB. The wildtype and mutant

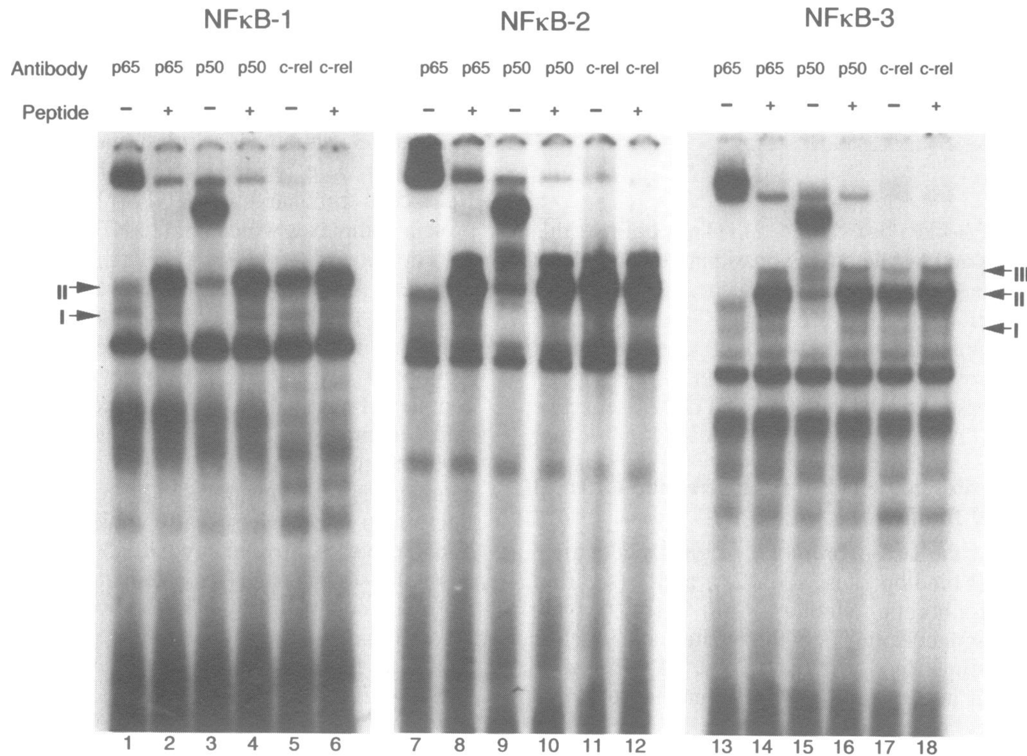

Figure 4. Identification of the protein complexes bound to NF-κB sites 1, 2 and 3. Antibodies to the p50 and p65 subunits of NF-κB and c-rel were used to identify the protein/DNA complexes bound to the putative NF-κB sites. Blocking peptides to these antibodies were used in blocking reactions to confirm the specificity of the antibodies. Nuclear extracts from TNFα stimulated HeLa cells were first incubated with the specified antibodies or pre-immune serum in the presence or absence of their blocking peptides. These reactions were then incubated with ^{32}P labeled probe to NF-κB sites 1(lanes 1–6), 2 (lanes 7–12), or 3 (lanes 13–18) and analyzed on a 0.25× Tris–borate EDTA native gels.

IκB-α promoter-CAT constructs were transfected into K562 cells and treated with TNFα, a known inducer of NF-κB activity. The wildtype promoter-CAT construct was inducible following TNFα stimulation (Fig. 6, lane 4). When NF-κB sites 1 and 2 were mutated, however, the IκB-α promoter-CAT construct was no longer responsive to TNFa stimulation which resulted in the decrease of CAT activity (Fig. 6, lanes 6 and 8). Mutating NF-κB site 3 also affected the ability of the IκB-α promoter-CAT construct to respond to TNFα (Fig. 6, lane 10), although mutating this site was not as detrimental as mutating NF-κB sites 1 or 2. These data demonstrate that NF-κB plays a role in regulating the IκB-α gene and that NF-κB sites 1 and 2 are required for TNFα-mediated gene activation, but NF-κB site 3 may play a lesser role in this response.

DISCUSSION

Previous studies have shown a correlation between loss of IκB-α protein in the cytoplasm and the reappearance of IκB-α message (14–17). This process presumably requires induced NF-κB binding activity. Other studies have shown that the p50 promoter is regulated by NF-κB (28,29). These data suggest that NF-κB and IκB-α are involved in an autoregulatory pathway to ensure the proper balance of NFκB and IκB-α in the cell. Our experiments here show direct interaction between NF-κB complexes and their target sites in the IκB-α promoter resulting in the activation of the IκB-α gene. We further show that there are three NF-κB sites in the IκB-α promoter which bind various p50, p65, and c-rel complexes in nuclear extracts from HeLa cells stimulated with TNFα and that mutating any one of these sites affects TNFα-mediated activation of the IκB-αa gene.

The NF-κB site 2 in this report has also been recently characterized in the porcine IκB-α promoter by de Martin *et al.* (30), in the murine promoter by Chiao *et al.* (31), and in the human promoter by Le Bail *et al.* (32). The latter group also identify another NF-κB site which corresponds to NF-κB site 3 in this report. In addition to these sites, we present additional sequence and characterize another NF-κB target sequence in the IκB-α promoter, NF-κB site 1, and show that it only binds p50 complexed with itself, p65, or c-rel from nuclear extracts of TNFα stimulated HeLa cells. The other two NF-κB sites 2 and 3, are able to bind to the same p50 protein complexes bound to NF-κB site 1 and also bind additional protein complexes predominantly comprised of p65 and c-rel homodimers or heterodimers. These data taken collectively indicate that the IκB-α gene is regulated by at least three fuctional NF-κB sites which have different affinities for various members of the Rel family. Since all three sites are essential for strong activation of the IκB-α gene following TNFα stimulation, there may be a cooperative functional interaction between the protein complexes bound to these sites.

We have shown that the IκB-α gene is regulated by NF-κB through at least three NF-κB sites, however, this gene may also be regulated by other transcription factors. There is an intriguing possibility that interactions between NF-κB and Spl might be involved in regulating this gene. An Spl site exists between NF-κB sites 1 and 2 (data not shown). Spl has been shown to interact with p65/p52 heterodimers on the HIV-1 LTR to activate transcription (33). The 3bp spacing between Spl and NF-κB sites in the HIV-1 LTR seems to be very important. The spacing between this Spl site and the NF-κB site 1 in the IκB-α promoter is similar to that in the HIV-1 LTR, suggesting a possible interaction between Spl and NF-κB protein complexes which bind to this site (33). The other NF-κB target sequence, site 2, is 10bp away from this Spl site. Since this distance has been shown to affect the transcriptional inducibility by these two transcription factors on the HIV-1 LTR (33), it seems unlikely that NF-κB bound to this site interacts with Spl to activate transcription of the IκB-α gene.

The NF-κB sites, the Spl site, and the putative TATA box as well as the IκB-α promoter as a whole are highly conserved between the porcine and the human IκB-α promoter (79.9% sequence homology). Homology decreases noticeably following an Alu repeat insertion in the porcine promoter at approximately −400 relative to the transcription start site identified here. There seems to be a discrepency as to where transcription of the IκB-α gene starts. We have mapped the transcription start site adjacent

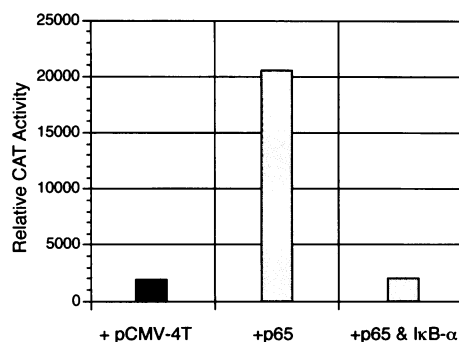

Figure 5. Regulation of the IκB-α gene by NF-κB. (A) HeLa cells were transfected using the calcium phosphate method with an IκB-α promoter-CAT construct plus a p65 expression vector alone, p65 and IκB-α expression vectors, or an empty expression vector, CMV-4T. 24 hours after transfection, cell extracts were harvested and CAT activity was determined. The fold induction is relative to the CAT activity of the CMV-4T empty vector. The relative CAT activities presented are representive of three independent experiments.

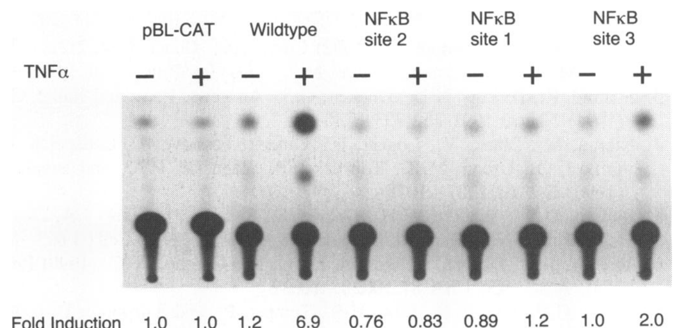

Figure 6. All three NF-κB sites in the IκB-α promoter are required for full activation of the IκB-α promoter-CAT construct following TNFa stimulation. K562 cells were transfected by electroporation with an IκB-α wildtype promoter-CAT construct or an IκB-α promoter-CAT construct containing mutations in either NF-κB sites 1, 2, or 3. 24 hours after transfection, TNFα (10 ng/ml) was added to the media and after another 24 hours, cell extracts were harvested. CAT activity was determined by thin layer chromotography and autoradiography. The fold induction was determined by densitometry and is relative to the CAT activity of the pBL-CAT vector in unstimulated cells which is arbitrarily set at 1.0. These values are an average of three independent experiments which did not vary from each other by more than 20%.

to a TATA-like box by primer extension using poly-A+ RNA from TNFα stimulated HeLa cells. LeBail *et al.* (32) use total RNA from PMA induced THP1 cells to identify two transcription start sites, one upstream and one downstream of the putative TATA-box. de Martin *et al.* (30) and Chiao *et al.* (31) likewise identify a single start site downstream of the putative TATA-box in the procine and murine IκB-α promoters, respectively. The start site mapped upstream of the TATA-like box and the various transcription start sites in the IκB-α promoter seem to be characteristic of a TATA-less promoter and allow for some speculation about the function of the TATA-like box. It seems plausible that the TATA-like box is not functional and that the IκB-α gene may be regulated by a TATA-less promoter or that there are different start sites depending on the inducer of IκB-α message and source of the RNA.

There also lies the possibility that other cis-acting elements may be required for the transcription of this gene. There is a putative NF-κB site in the first intron of the IκB-α gene (manuscript in prep). This putative NF-κB site in the first intron is identical to one of the NF-κB sites found in the HIV-1 LTR (9). It is possible that the NF-κB sites in the first intron may act as enhancers and may augment the induction of this gene by TNFα or other cytokines. Although further studies need to be done in order to fully understand the roles of other transcription factors and cis elements in the regulation of the IκB-α gene our studies show that NF-κB and Rel proteins regulate IκB-α gene expression.

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