Requirement for Nuclear Factor (NF)-κB p65 and NF-Interleukin-6 Binding Elements in the Tumor Necrosis Factor Response Region of the Granulocyte Colony-Stimulating Factor Promoter

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Granulocyte colony-stimulating factor (G-CSF) is a hematopoietic growth factor produced by mesenchymal and myeloid cells following activation by inflammatory stimuli. It has previously been shown that a region of the G-CSF promoter, (~200 to −165) containing the decanucleotide CK-1 element and two repeated sequences that resemble nuclear factor (NF)-interleukin-6 (IL-6) binding sites, is required for activation of the G-CSF gene by tumor necrosis factor-α (TNF-α) and IL-1β. We now show that the NF-κB p65 protein can bind to and activate this TNF response region. There are several unusual features of this p65 interaction with the TNF response region. First, NF-κB p65 but not the related NF-κB p50 binds to the CK-1 element and a p50/p65 hybrid protein that relies on the p65 rel homology domain for DNA binding does not transactivate the TNF response region. Second, p65 transactivation of this region is cell specific and requires not only its own binding site but also the NF-IL6 consensus sites. NF-IL6 also binds to the TNF response region of the G-CSF promoter. Electrophoretic mobility shift studies show that p65 and NF-IL6 can bind cooperatively to the TNF response region. The ability of this region to respond to TNF-α or p65 is correlated with the ability to form the p65/NF-IL6 ternary complex.

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Submitted July 13, 1993; accepted January 3, 1994.

Supported by funds from the National Health and Medical Research Council of Australia. S.M.D. was supported by a postgraduate scholarship from the National Heart Foundation, Australia.

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0006-4971/94/8309-0021$3.00/0


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sequences and pIgM(5), all cloned upstream of the 2(5+) containing the GM-CSF CK-1 element and surrounding sequences pCK-1(4+) containing the G-CSF TNF response region, pCK-11 excised from Bluescript clones using Nor I and cloned into a Not I CAT2, have been previously described." (Fig 1). Sequences of the phosphorylated and annealed to yield double stranded DNA." The inserts were excised with BamHl/HindIII and subcloned into pBLA, which contains the basal thymidine kinase promoter,22 pCK-1(4+) containing the G-CSF TNF response region, pCK-1/ 2(5+) containing the GM-CSF CK-1 element and surrounding sequences and pgex(5), all cloned upstream of the tk promoter in pBL CAT2, have been previously described15,16 (Fig 1). Sequences of the oligonucleotides used to generate these reporters is shown in Fig 1. pCK-1M5(3) has three copies of oligonucleotides with a 3-bp mutation in the CK-1 element (Fig 1), pCK-1M(5) contains five copies with a 3-bp mutation in the second NF-IL6 consensus element, pGM/G(3) and pG/GM(3) have three copies of hybrid GM-CSF and G-CSF CK-1 regions (Fig 1).

Full-length cDNAs for murine NF-κB p50 and p65 were excised from Bluescript clones using Not I and cloned into a Not I linearized Rc/CMV expression vector (Invitrogen Corp, San Diego, CA) to generate CMVp565(+) and CMVp50(+). An Apa I fragment of the p50 cDNA encoding the first 464 amino terminal amino acids was also cloned into Rc/CMV (CMVp50A). A CMV-based expression plasmid containing a hybrid cDNA for the rel homology domain of p50 (amino acid 1-370) and the transactivation region of p65 (amino acids 309-550) was a gift from C. Kunsch (Roche Institute of Molecular Biology, Nutley, NJ).

Cells and transfection. Human embryonic lung fibroblasts (Commonwealth Serum Laboratories, Melbourne, Australia) were maintained and transfected with diethyl aminoethyl dextran as previously described.18 For cotransfection experiments 5 μg of reporter and 10 μg of expression plasmid were used. The Jurkat T lymphoblastoid cell line was also maintained and transfected by electroporation as previously described.17 For cotransfections in Jurkat cells, 1 μg of reporter and 5 μg of expression plasmid was used. HEL fibroblasts were treated with TNF-α and IL-1β, cell extracts prepared and chloramphenicol acetyl transferase (CAT) assays performed as previously described.18 Percentage CAT conversion was determined using phosphorimage analysis (Molecular Dynamics, Inc, Sunnyvale, CA). Relative CAT activity was determined within each experiment using a value of one for the unstimulated pCK-1(+4+) transfection.

Production of recombinant proteins. An Fsp VXho I fragment of p50 cDNA35 encoding 498 amino acids was subcloned into Bluescript KS(+) plasmid DNA was linearized with HindIII, to generate a truncated protein of 363 amino acids, and capped RNA transcripts generated using T7 RNA polymerase (Promega). Full length NF-IL6 cDNA from a CMV NF-IL6 clone13 (gift from S. Akira) was subcloned into pSP72, linearized with EcoRI and transcribed with SP6 polymerase. RNA was translated using wheat germ extract (Promega, Madison, WI) that was pre cleared for endogenous DNA binding proteins with GM-CSF CK-1 oligonucleotides coupled to sepharose beads. A fragment of p65 cDNA42 encoding amino acids 1-313 was generated by PCR and subcloned in frame with the glutathione S-transferase protein into pCex-2T. The GST-p65 fusion protein was produced, purified, and cleaved with thrombin as described.35

DNA binding assays. Protein-to-DNA binding assays for p50 contained 2 μl in vitro translated protein, 2 μg polydI:dC, 3 mmol/L GTP, 0.2 ng radiolabeled double stranded oligonucleotide (5 to 10,000 cpm), TM buffer containing 50 mmol/L KC1 in a final volume of 20 μL. The reactions were analyzed on 5% polyacrylamide gels containing 50 mmol/L Tris pH 8.5, 380 mmol/L glycine, and 2 mmol/L EDTA. NF-IL6 and p65 binding reactions were performed in 20 mmol/L HEPES pH 7.9, 50 mmol/L KC1, 1 mmol/L EDTA, 0.5 mmol/L dithiothreitol. Reactions were separated on a 5% polyacrylamide gel in 12.5 mmol/L Tris pH 8.3, 10.5 mmol/L boric acid, 0.25 mmol/L EDTA. For mixed binding experiments, NF-IL6 and p65 were preincubated for 10 minutes at room temperature before the addition of the radiolabeled DNA. Antibodies were preincubated for 5 minutes at room temperature, with the appropriate proteins, before being added to the binding reactions. To determine the binding kinetics of individual proteins and protein complexes, a

Fig 1. Sequences of oligonucleotides used in gel shift assays and reporter constructs. The CK-1 element and the direct repeat NF-IL6 consensus elements (**) across these sequences are indicated. The altered bases in each mutant oligonucleotide are shown. The name of the CAT reporter construct (the number of copies in parentheses) derived from each oligonucleotide is indicated.
The NF-κB p65 protein transactivates the TNF response region of the G-CSF promoter. A reporter plasmid containing four copies of a 40-bp sequence spanning the CK-1 element and the repeated NF-IL6 consensus elements of the G-CSF promoter, pCK-1(4+), was previously shown to be TNF-α and IL-1β responsive in human embryonic lung fibroblasts.18 Because we observed that the CK-1 element matches the p65 homodimer consensus binding site, we examined whether overexpression of NF-κB proteins could also activate the TNF response region of G-CSF. Overexpression of NF-κB p65 transactivated pCK-1(4+) in HEL fibroblasts (average 17-fold) (Fig 2A). Overexpression of NF-κB p50, either full-length (Fig 2A) or truncated forms (data not shown), did not activate pCK-1(4+). Cotransfection of p50 with p65 gave the same results as p65 alone (data not shown). A well-characterized NF-κB site from the immunoglobulin (Ig)κ gene [pIgκ(5)] was also transactivated (average sixfold) by p65 but not p50 overexpression in HEL fibroblasts (Fig 2A). TNF-α treatment of HEL fibroblasts also induced expression from transfected pCK-1(4+) and pIgκ(5) (Fig 2A) as previously described.18 Because p50 has previously been shown to lack transactivation ability following transfection, we used a p50/65 hybrid protein construct, which is dependent on the p50 rel homology domain for DNA binding but uses the transactivation domain of p65.
The hybrid p50/65 construct transactivated plgκ(5) (18.4-fold, average of four experiments) but not pCK-1(4+) in HEL fibroblasts (Fig 2B). In this set of experiments, pCK-1(4+) was not as well activated by p65 compared with Fig 2A (sevenfold compared with 17-fold) but nevertheless no activation by the p50/65 hybrid protein was detected. The p50/65 hybrid protein activated the Igκ sequence better than did p65 alone indicating its function in these cells.

The cell specificity of p65 transactivation of the G-CSF TNF response region was tested in Jurkat T cells where G-CSF is not normally expressed. pCK-1(4+) was only weakly responsive (fourfold) in Jurkat T cells whereas plgκ(5) was strongly activated by p65 (85-fold) (Fig 2C). Overexpression of p50 gave low-level activation of plgκ(5) (fivefold) but not pCK-1(4+) (Fig 2C).

NF-κB p65 transactivation and TNF-α induction of the CK-1 region have similar sequence requirements. We have previously shown, either in the context of the intact G-CSF promoter17 or the pCK-1(4+) construct,18 that both the CK-1 decanucleotide element and the second NF-IL6 consensus element are required for TNF-α/IL-1β activation. We have also shown that the CK-1 element in the context of the GM-CSF promoter is not responsive to TNF-α/IL-1β.26 To determine whether p65 transactivation and TNF-α activation have the same sequence requirements, we compared the response of a series of constructs containing intact or mutant G-CSF or GM-CSF sequences with p65 overexpression or TNF-α treatment. Mutations either in the CK-1 element (M5) or in the second NF-IL6 consensus element (M6) abolished both the response to p65 and TNF-α (Fig 3). The GM-CSF CK-1 construct (pCK-1/2(5+)) also did not respond to either p65 or TNF-α (Fig 3).

The GM-CSF CK-1 region contains one potential NF-IL6 site overlapping the CK-1 element (see Fig 1) but does not have the second repeat element. To determine if the GM-CSF CK-1 element could function with two intact NF-IL6 consensus elements, we generated a hybrid construct (pGM/G(3)) containing the 5′ end of the GM-CSF sequence linked to the 3′ end of the G-CSF sequence whereby the CK-1 element and its 5′ flanking sequence were contributed by GM-CSF and the second NF-IL6 consensus element by G-CSF (Fig 1). The reciprocal plasmid pGM/G(3) was also constructed (Fig 1). p65 overexpression transactivated pGM/G(3) but not pG/GM(3) (Fig 3) implying that the CK-1 element was functional only when linked to an additional NF-IL6 element. TNF-α also activated pGM/G(3) but not pG/GM(3) (Fig 3). These data show that sequences other than the CK-1 p65 consensus binding site are required for p65 transactivation of the TNF response region and that p65 transactivation parallels the TNF-α response.

The NF-κB p65 protein and NF-IL6 both bind to the G-CSF TNF response region. The ability of p65 and p50 NF-κB proteins and NF-IL6 to bind DNA fragments spanning the G-CSF and GM-CSF CK-1 elements was tested. The p50 protein did not bind to either the G- or GM-CSF elements but did generate a complex with the Igκ NF-κB element (Fig 4A). p50 also bound to an extended GM-CSF oligonucleotide that contained a previously described NF-κB site17 downstream of CK-1 (Fig 4A). The specific p50 complex was identified by inclusion of an anti-p50 antibody in the binding reactions. This antibody but not an anti-p65 antibody abolished the complex marked as p50 (Fig 4B). The specificity of this complex was also determined by competition experiments (data not shown). All the other bands seen in the gel shift lanes are the binding of endogenous nonspecific proteins from the wheat germ extracts. The lack of p50 binding to the G-CSF sequence correlates with the failure of the p50/65 hybrid protein to transactivate these sequences. In contrast, NF-κB p65 binding was detected to both the G- and GM-CSF DNA fragments as well as to the Igκ NF-κB site (Fig 4C). Two complexes were detected with both the G- and GM-CSF probes. The upper complex could be com-
Fig 4. NF-κB p65 but not p50 binds to the G-CSF and GM-CSF CK-1 regions. (A) Binding of in vitro synthesized p50 to radiolabeled Igκ (lane 1), G (lane 2), and GM (lane 3) DNA fragments (see Fig 1) and GM2 (lane 4) (NF-κB site in the GM-CSF promoter [5]). (B) p50 binding to Igκ (lane 1) with no antibody added to the binding reactions. Anti-p65 (lane 2) or anti-p50 (lane 3) antibodies were added to the protein in the binding reactions 10 minutes before addition of the radiolabeled probe. (C) Binding of NF-κB p65 to radiolabeled Igκ (lanes 1-3), G (lanes 4-6), and GM (lanes 7-9) oligonucleotides (0.2 ng). Competitors (20 ng; 100-fold excess) used were Igκ(I) and a mutant Igκ(M) sequence (see Fig 1). (D) p65 binding to both G and GM probes was inhibited by addition of an anti-p65 antibody (1 μL) (lanes 2 and 4) but not a p50 antibody (1 μL) to the binding reactions.
detected, (marked x), migrating above the NF-IL6 band (Fig 6A). Binding reactions, in which one of the protein components was titrated with a fixed amount of the second component, were carried out to determine if cooperative binding was occurring. At a fixed concentration of p65, increasing amounts of NF-IL6 leads to an increase in complex x and a decrease in the p65 complex (Fig 6B). Phosphorimager quantitation of these experiments showed that complex x formed at least three times as efficiently as the NF-IL6 complex alone. Therefore, it appears that the formation of the complex x is favored over the binding of individual proteins.

Antibodies to p65 and NF-IL6 were used to determine the components of complex x. An antibody to p65 abolished p65 binding to the CK-1 region (see Fig 4D) but did not affect NF-IL6 binding (Fig 6C). However, in p65/NF-IL-6 mixing experiments this antibody abolished not only the p65 complex but also the x complex (Fig 6C). A complex that migrates slightly below the x complex was detected in some p65/NF-IL6 mixing experiments (see Fig 6C and D). This complex (referred to as x') in Fig 6C always migrated faster than the NF-IL6 alone complex and was always abolished by the anti-p65 antibody (Fig 6C). The nature of this complex is unclear. An antibody to p50 had no effect on any of the complexes (Fig 6C). In mixing experiments the C/EBP-β antibody eliminated both the x and faster migrating complexes but did not affect p65 binding (Fig 6B). The C/EBP-α antibody reduced the intensity of these complexes (Fig 6C). The C/EBP-α antibody was raised against the entire protein and, therefore, cross-reactivity with the related NF-IL6 protein is not surprising. Because complex x is abolished by antibodies to both NF-IL6 and p65 it implies that the complex represents simultaneous binding of both proteins to a single DNA fragment. Competition experiments using competitor oligonucleotides that bound only one or other of the proteins also indicated that complex x contained both p65 and NF-IL6 (Fig 6D).

The ability of mutant sequences across the TNF response region were tested for their ability to bind NF-IL6, p65 and the NF-IL6/p65 complex. Three mutants were chosen for this analysis; M8 has a mutation at the 5' end of the CK-1 element, which we have shown to abolish p65 binding in the GM-CSF context and p65 transactivation of the GM/G hybrid (unpublished data). M5 and M6 have been shown here to abolish TNF and p65 response of the G-CSF sequence. M8 maintains NF-IL6 binding but has lost the capacity to bind p65 and form the p65/NF-IL6 complex (Fig 7A). Conversely, M6 binds p65 but does not bind NF-IL6 nor the p65/NF-IL6 complex (Fig 7A). M5 binds neither p65 nor NF-IL6 (Fig 7A). These data show that the binding sites for both proteins must be intact to generate the p65/NF-IL6 complex and function. Because the G-CSF TNF response region and the GM/G hybrid but not the GM-CSF CK-1 region were transactivated by p65 (see Fig 3), we tested the ability of these sequences to bind p65 and NF-IL6 individually and to form complex x. Both G and GM/G sequences could bind NF-IL6 weakly but the GM sequence did not (Fig 7B). The faint band migrating in the region of NF-IL6 with the GM-CSF probe is nonspecific and found in wheat

**Fig 5.** NF-IL6 binds to the G-CSF TNF response region. In vitro synthesized NF-IL6 forms a complex with the G (lanes 2-4) and IL-6 (lanes 6-8) radiolabeled probes, which can be abolished by the addition of an anti-CIEBP antibody (lanes 3 and 7) but not by the addition of an antibody to the NF-κB p50 protein (lanes 4 and 8). Lanes 1 and 5 had unprimed wheat germ extract added to the binding reactions. The binding of NF-IL6 to the G probe (0.2 ng) (lane 5) was eliminated by the addition of 10 ng (50-fold excess) of unlabeled wild type IL-6 oligonucleotide (lane 10) (see Fig 1) but not by the addition of 10 ng of mutant IL-6 oligonucleotide (lane 11).
Fig 6. NF-IL6 and NF-κB p65 can bind simultaneously to the G-CSF TNF response region. (A) NF-IL6 (lane 1), p65 (lane 2), and the NF-IL6/p65 mixture (lane 3) were tested for binding to the G-CSF TNF response region. The NF-IL6 and p65 proteins were mixed and incubated for 10 minutes before addition of the radiolabeled probe. (B) Increasing amounts of wheat germ extract primed with NF-IL6 RNA (shown in microliters) either alone (lanes 2-6) or preincubated with a fixed amount of p65 (lanes 7-11) were mixed with the radiolabeled G-CSF probe and analyzed on a 6% nondenaturing polyacrylamide gel. p65 alone with the radiolabeled probe (lane 1) is also shown. The NF-IL6 and p65 alone as well as the p65/NF-IL6 ternary complex (marked as x) are indicated. (C) An anti-p65 antibody did not affect NF-IL6 binding (lane 2) but did abolish complex x (lane 6). Antibodies to C/EBP α and β both reduced complex x formation but not p65 binding (lanes 14 and 15). The anti-p50 antibody had no effect on the complex (lane 7). (D) Binding sites for both NF-IL6 and NF-κB proteins can compete for complex x formation. Increasing concentrations (shown in nanograms; fivefold to 100-fold excess) of the IL-6 (lanes 2-5) and Igκ (lanes 6-9) oligonucleotides were added to binding reactions with the G radiolabeled probe, NF-IL6 and p65 proteins. Lane 1 had no competitor added.
germ extracts without NF-IL6 mRNA (data not shown). All three DNA fragments bind p65 with strongest binding observed to the GM-CSF sequence (Fig 7B—compare with Fig 4C). More importantly, complex x was seen when p65 and NF-IL6 were mixed and tested for binding to the hybrid GM/G sequence but was not observed with the GM sequence (Fig 7B). Neither the p65 complex nor complex x were detected with the NF-IL6 site from the IL-6 gene (Fig 7B). These results show that the putative p65/NF-IL6 complex x is only detected with oligonucleotides that bind both NF-IL6 and p65 independently. The ability to form this complex also correlates with the ability of these sequence elements to be activated by TNF-α/IL-1β and to respond to p65 overexpression.

**DISCUSSION**

We have shown here that NF-κB p65 binds to and trans-activates the TNF response region of the G-CSF promoter. The unique feature of this transactivation is that it appears to be dependent on the simultaneous binding of NF-κB p65 and NF-IL6 or related C/EBP proteins to this region. The observation that NF-κB p65 bound to the G-CSF TNF response region but that NF-κB p50 did not is in contrast to most NF-κB sites, which can generally bind p50 more avidly than p65. A 7 and 8 out of 10 match to the recently defined p65 consensus can be found across the CK-1 element of G-CSF and GM-CSF, respectively (Fig 8A), whereas the match to the p50 consensus is only 4 and 5 out of 10. The potential p65 binding site lies across the 5′ end of the CK-1 element (Fig 8A) and mutations across this region in G-CSF (Fig 6D) in GM-CSF have confirmed that this region is required for p65 binding. We have previously shown that NF-κB purified from bovine spleen did not bind to the G-CSF TNF response region nor the GM-CSF CK-1 element. This purified material was enriched for NF-κB p50 and so p65 binding may not have been detectable. Binding of p50/p65 heterodimers or NF-κB from crude nuclear extracts was also not detected to the G-CSF TNF response region (unpublished observations). The CK-1 element also binds another TNF-α inducible protein, NF-GMa. Antibodies against NF-κB or C/EBP proteins do not affect NF-GMa binding (unpublished observation). Whether NF-GMa can bind together with p65 or NF-IL6 and its role in activation of this region is still unclear.

NF-IL6 binding to the TNF response region was also detected. Recently, Nishizawa et al cloned another C/EBP protein using this G-CSF promoter fragment as a probe. We have not mapped the exact binding site for NF-IL6 but the noncoding strands of the direct repeats of the direct repeats both match the NF-IL6 consensus sequence (Fig 8B). It is clear that both repeated elements are required for function because all the mutations shown here to abolish function lie within these repeats (see Fig 1). The M5 but not the M6 mutation abolishes p65 binding (Fig 7A). The lack of M6 function cannot be accounted for by lack of p65 binding and may be associated with the abolition of an NF-IL6 binding site. Methylation-inhibition experiments will be required to determine the precise binding sites for NF-IL6 and also p65.

Although p65 can bind to a number of DNA fragments...
ACTIVATION OF G-CSF BY NF-κB AND NF-IL6

A

p65 CONSENSUS

G G G R N T T T C C

G-CK-1 C A G A G A T T C C 7/10

GM-CK-1 A G A G A G A T T C C 8/10

NF-IL6 CONSENSUS

A T T N N G N A A T

G 1 A T T C C A C A A T 9/10

G 2 A T T T C A C A A A 8/10

Fig 8. (A) Comparison of the G- and GM-CSF CK-1 elements with the p65 consensus sequence from Kunsch et al. Identical bases to the consensus are indicated by stars. (B) Comparison of the repeated NF-IL6 elements of the TNF response region of G-CSF with an NF-IL6 consensus sequence.

containing CK-1 elements, transactivation only occurs when the DNA fragment can also bind NF-IL6. This is in contrast with most of the previously described NF-κB sites, which function independently. Other genes have been shown to have functionally linked NF-IL6 and NF-κB sites. In the IL-6 gene promoter NF-κB and NF-IL6 sites, which are 70 bp apart, are required for activity but can function independently. The PCR-based screen for p65 binding sites, discussed above, detected the NF-κB sites in both the angiotensinogen and the IL-8 gene promoters. This is intriguing because these promoters both have overlapping or adjacent NF-κB and NF-IL6 sites located in functional promoter regions. Those sequences in the PCR screen that bound p65 but not p50 homodimers also did not bind p50/p65 heterodimers and it was proposed by Kunsch et al that p65 homodimers and not p50/p65 mediated the activity of the p65-specific binding sites. We have shown that a p50/65 hybrid protein, the DNA binding of which is mediated by p50, does not transactivate the G-CSF TNF response region but does transactivate the Igκ NF-κB site. The result supports the lack of p50 binding in vitro by showing that a protein dependent on p50 sequences for DNA binding cannot transactivate this region in vivo. It is possible that p65 homodimers activate the G-CSF TNF response region but that efficient activation requires the simultaneous binding of NF-IL6 or related C/EBP proteins.

There appears to be cooperative binding of p65 and NF-IL6 to the TNF response region of G-CSF. The formation of this ternary complex correlates with the function of this region. Mutations that abolish complex formation but allow binding of one or other of the proteins are not active. Furthermore, the G-CSF and GM/G sequences that can form the ternary complex are p65 or TNF-α responsive, whereas the GM-CSF sequence that binds p65 but not the ternary complex is not activated by either p65 or TNF-α. We have not determined whether direct protein-to-protein contact occurs between NF-IL6 and p65 although there is indirect evidence that this occurs. Most of the DNA binding experiments were carried out with an excess of NF-κB p65 binding activity over NF-IL6 binding activity. When an antibody against NF-κB p65 is included in the binding reactions, the p65/NF-IL6 complex as well as the p65 complex were eliminated but the NF-IL6 complex did not reappear suggesting that all the NF-IL6 was sequestered by antibody-bound p65 in solution. If the quantity of p65 in the binding reactions is reduced then the anti-p65 antibody eliminates the p65 and x complexes but does not effect the NF-IL6 complex (data not shown). LeClair et al detected an interaction between NF-κB p50 and NF-IL6 in the absence of DNA but could not detect the interaction of p50 and NF-IL6 with the same DNA fragment. More recently, a physical and functional association has also been shown between NF-κB p50 and p65 with C/EBPβ. This functional interaction results in the inhibition of κB site containing promoters and the synergistic stimulation of C/EBP binding promoters. Again, there was no evidence for the direct interaction of both proteins with the same DNA sequence. The results presented here show for the first time a cooperative direct DNA binding interaction of NF-κB and C/EBP proteins.

It is likely that NF-κB p65 is required for TNF-α/IL-1β activation of the G-CSF promoter because TNF-α activation has sequence requirements identical to those of p65 transactivation. In addition, the expression of antisense NF-κB RNAs can block the TNF-α–mediated activation of the CK-1 region (unpublished observations). TNF-α activation has also been shown to upregulate NF-IL6 function. It is likely that the expression and function of both NF-κB p65 and NF-IL6 is required for TNF-α/IL-1β activation of the G-CSF promoter. It is clear from our data that only those sequences that bind both NF-κB p65 and NF-IL6 are functional. How-
ever, transfection experiments with both NF-κB p65 and NF-IL6 have not shown increased levels of expression over those with NF-κB p65 alone. There are at least two possible explanations for this observation. First, it has been shown that overexpressed NF-IL6 requires posttranslational modification for function. However, TNF-α treatment of cells that have been transfected with RC/CMV NF-IL6, also failed to give increased levels of transactivation (unpublished observations). Second, it is possible that the HEL fibroblasts used in these experiments already contain sufficient quantities of C/EBP proteins that can cooperate with the overexpressed NF-κB p65 to mediate activation.

Expression of genes such as G-CSF and IL-8 involved in inflammatory responses may be dependent on interaction between NF-κB and NF-IL6, generating a subset of genes with a narrower range of response than those genes activated by NF-κB or NF-IL6 alone. This is borne out by our experiments in Jurkat T cells where NF-κB can be induced but NF-IL6 is not expressed. Neither the TNF response region, the 330-bp G-CSF proximal promoter, nor the endogenous G-CSF gene are functional in these cells. NF-κB p65 cannot efficiently transactivate the G-CSF TNF response region in Jurkats where it gives high level transactivation of the Igκ NF-κB site. It will be of interest to determine the general role of NF-κB/NF-IL6 interactions in the expression of inflammatory cytokines.

ACKNOWLEDGMENT

We thank Anna Sapa for excellent technical assistance, Mari Walker, Annette Davies, and Angie Elekes for secretarial services, and Dr Peter Cockerill and Dr Mike Lenardo for critical comments.

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Requirement for nuclear factor (NF)-kappa B p65 and NF-interleukin-6 binding elements in the tumor necrosis factor response region of the granulocyte colony-stimulating factor promoter

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