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NF-κB as Inducible Transcriptional Activator of the Granulocyte-Macrophage Colony-Stimulating Factor Gene

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The expression of the gene encoding the granulocyte-macrophage colony-stimulating factor (GM-CSF) is induced upon activation of T cells with phytohemagglutinin and active phorbolester and upon expression of tax_1, a transactivating protein of the human T-cell leukemia virus type I. The same agents induce transcription from the interleukin-2 receptor α-chain and interleukin-2 genes, depending on promoter elements that bind the inducible transcription factor NF-κB (or an NF-κB-like factor). We therefore tested the possibility that the GM-CSF gene is also regulated by a cognate motif for the NF-κB transcription factor. A recent functional analysis by Miyatake et al. (S. Miyatake, M. Seiki, M. Yoshida, and K. Arai, Mol. Cell. Biol. 8:5581–5587, 1988) described a short promoter region in the GM-CSF gene that conferred strong inducibility by T-cell-activating signals and tax_1, but no NF-κB-binding motifs were identified. Using electrophoretic mobility shift assays, we showed binding of purified human NF-κB and of the NF-κB activated in Jurkat T cells to an oligonucleotide comprising the GM-CSF promoter element responsible for mediating responsiveness to T-cell-activating signals and tax_1. As shown by a methylation interference analysis and oligonucleotide competition experiments, purified NF-κB binds at positions −82 to −91 (GGGAACTACC) of the GM-CSF promoter sequence with an affinity similar to that with which it binds to the biologically functional κB motif in the beta interferon promoter (GGGAAATTCC). Two κB-like motifs at positions −98 to −108 of the GM-CSF promoter were also recognized but with much lower affinities. Our data provide strong evidence that the expression of the GM-CSF gene following T-cell activation is controlled by binding of the NF-κB transcription factor to a high-affinity binding site in the GM-CSF promoter.

The NF-κB transcription factor has been implicated in the transcriptional activation of viruses and a variety of genes involved in immune and inflammatory responses (for a review, see reference 16). A characteristic of NF-κB is that it exists in a latent form in the cytoplasm of cells and thus not only is rapidly activated upon stimulation of cells without de novo synthesis but also can participate in signaling between the cytoplasm and nucleus (1–3). The inactive form of NF-κB is apparently stabilized by a specific inhibitory protein designated IκB (2). Deoxycholate (DOC) can dissociate the inactive complex of NF-κB and IκB in vitro, thereby activating NF-κB. In intact cells, a large number of agents were found to activate NF-κB, among them 12-O-tetradecanoylphorbol 13-acetate (TPA), lipopolysaccharide, lectins, double-stranded RNA, interleukin-1 (IL-1), tumor necrosis factor α, cyclic AMP, the tax_1 protein encoded by human T-cell leukemia virus type I, and the X protein of hepatitis virus type B (16 and references therein). It is unclear how the distinct intracellular signals elicited by these agents funnel into the NF-κB–IκB complex, resulting in its dissociation.

NF-κB, a widely used mediator of immediate-early gene expression (15, 16), is apparently also used in T lymphocytes to rapidly activate genes following T-cell activation. Three well-studied examples are the IL-2 receptor (IL-2R) gene (4, 5, 6, 8, 17), the IL-2 gene (13), and the human immunodeficiency virus type 1 (12, 22). In all three systems, the *cis*-acting elements that confer inducibility to the respecti promoters by T-cell-activating signals were shown to be binding sites for the NF-κB transcription factor. Also, the tax_1 protein from human T-cell leukemia virus type I has been shown to activate genes via binding sites for NF-κB (4, 11, 17). Mutations in the κB sites that prevent factor binding abolished the activities of the *cis*-acting DNA elements.

The granulocyte-macrophage colony-stimulating factor (GM-CSF) gene was previously shown to be activated by a TPA-phytohemagglutinin (PHA) or a TPA-calcium ionophore treatment mimicking T-cell activation and upon transient expression of tax_1 (7, 19, 20, 23). In a deletion and mutation analysis of the mouse GM-CSF promoter region, two short sequence elements between positions −113 and −73 upstream of the transcriptional start site that were sufficient to confer inducibility to the chloramphenicol transferase (CAT) gene were identified (20). An element between positions −95 and −73 conferred a strong response to T-cell-activating signals as well as to transient expression of tax_1 (Fig. 1A). A second element responding only weakly to transient tax_1 expression but not to T-cell-activating signals was located between positions −113 and −95. Although no protein-binding data were obtained in this study, the *cis*-acting effects were attributed to conserved lymphokine elements designated CLE 1 and CLE 2 and a GC box present within the segment (Fig. 1A). Because NF-κB-binding sites were shown to confer transcriptional responses, as those found with the GM-CSF promoter elements, we investigated whether the GM-CSF gene is regulated by this transcription factor. Here, we show that a high-affinity binding site for the NF-κB transcription factor is present in the *cis*-acting element conferring strong inducibility upon T-cell activation and tax_1. The CLE 1 motif which confers only weak inducibility by tax_1 is a low-affinity binding site for NF-κB. Combined with the data from the deletion and mutation analysis of the GM-CSF promoter (20, 23), our data strongly

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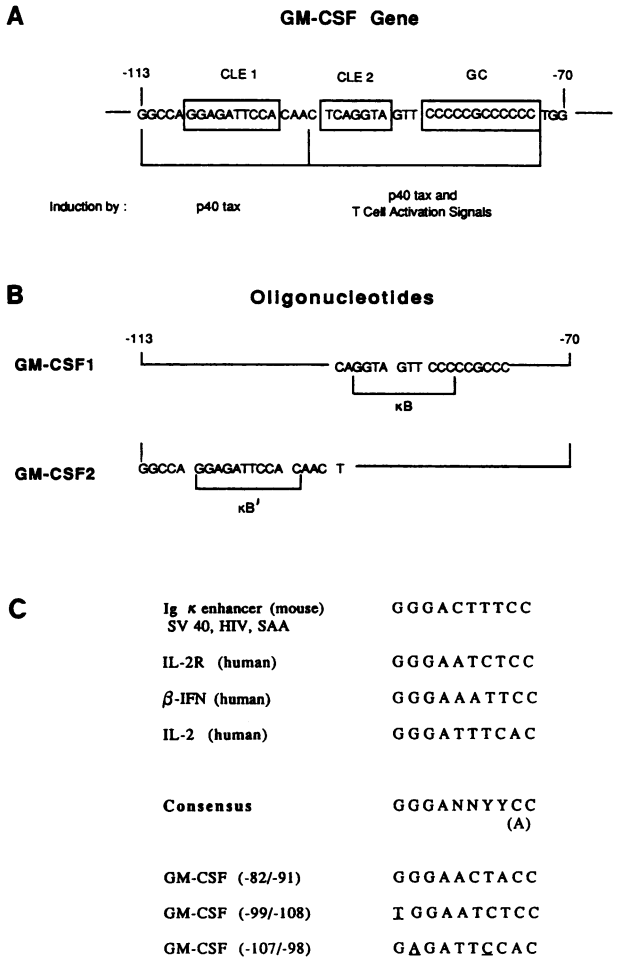

FIG. 1. Putative NF-κB-binding sites in the GM-CSF promoter. (A) Schematic representation of a segment of the mouse GM-CSF upstream promoter as described by Miyatake et al. (20). The sequence motifs CLE 1, CLE 2, and the GC box and their proposed functional significance in inducible expression of the GM-CSF gene are indicated. (B) Oligonucleotides used in this study. Apart from the sequences that are shown in alignment with that in panel A, the double-stranded oligonucleotides contained *Hin*dIII and *Sal*I linker sequences on 5′ and 3′ ends, respectively. The positions of putative NF-κB binding sites (κB and κB′) are indicated by brackets. (C) Alignment of the κB motifs in the GM-CSF promoter with physiological NF-κB-binding sites. κB motifs that were shown to have functional significance for inducible gene expression (16 and references therein) are aligned, and a consensus sequence is shown (N, any nucleotide; Y, pyrimidines). Only the strand that contains the three clustered conserved guanosine residues whose methylation interferes with factor binding is depicted. Three κB-like elements of the GM-CSF promoter are shown, and their positions in the promoter are indicated with reference to the sequence in panel A. T, Deviation from the human IL2-R site; A and C, deviations from the human IL-2 site; Ig, immunoglobulin; SV40, simian virus 40; HIV, human immunodeficiency virus; β-IFN, beta interferon; SAA, serum amyloid A.

suggest that NF-κB is a transcriptional activator of the GM-CSF gene.

Putative NF-κB binding sites in the upstream promoter region of the GM-CSF gene. We identified by inspection three putative binding sites for NF-κB within promoter elements of the GM-CSF gene that were shown earlier to confer inducible expression by T-cell activation signals and tax_1 when inserted into constructs containing the bacterial CAT gene (20). One potential NF-κB-binding site is located on the noncoding strand overlapping the sequence elements designated CLE 2 and GC (Fig. 1A). This site has the sequence GGGAACTACC and differs only by a purine in position 8 from the consensus sequence GGGANNYYCC that was delineated by comparing biologically functional NF-κB-binding sites (Fig. 1C). Two other κB-like sequences are present in the sequence element designated CLE 1 (Fig. 1A and C), which is conserved among promoters of many cytokine genes (for a list of such genes, see reference 26). A putative site on the coding strand has the sequence GAGAT TCCAC and differs by an A in position 2 and by a C in position 7 from the functional NF-κB-binding site found in the IL-2 gene (13). Another site on the noncoding strand has the sequence TGGAATCTCC and deviates from the functional site found in the promoter of the human IL-2R α-chain (5) gene only in position 1 (Fig. 1C).

High-affinity binding of purified NF-κB to a site in the GM-CSF promoter. To examine whether NF-κB would bind to the putative sites in the GM-CSF promoter with an affinity comparable to that of biologically functional NF-κB-binding sites, we performed an analysis comprising electrophoretic mobility shift assays (EMSAs) and oligonucleotide competitions. Four double-stranded 34-mer oligonucleotides containing in their centers the NF-κB-binding sites found in the IL-2R and IL-2 promoters and the putative sites of the GM-CSF promoter (Fig. 1B), respectively, were labeled with [α-^{32}P]dCTP by the Klenow enzyme to the same specific activity and reacted with equal amounts of NF-κB purified from the cytosol of human placenta. EMSAs were essentially performed as described previously (25). A typical binding reaction contained 1 to 2 μg of poly(dI-dC) (Pharmacia), 5,000 to 10,000 cpm (Cerenkov counting) of ^{32}P-labeled DNA probe, 2 μl of buffer D (9) containing 1% (vol/vol) Nonidet P-40 (Sigma Chemical Co.), 20 μg of bovine serum albumin, and binding buffer (25). The amount of purified NF-κB used (Fig. 2A) was about 50 pg per assay. The NF-κB was purified from the cytosol of human placenta (U. Zabel and P. Baeuerle, manuscript in preparation) and consisted of a 50-kilodalton (kDa) and a 65-kDa polypeptide, as recently described for the NF-κB from cytosol of HeLa cells (2a) and as observed with the nuclear NF-κB from human B cells (14).

The oligonucleotide GM-CSF1 (Fig. 1B) was as strongly complexed by NF-κB as were the IL-2R and IL-2 oligonucleotides (Fig. 2A). Also, the GM-CSF2 probe was bound by NF-κB but gave rise to less than 10% of the complex obtained with the other probes. In a subsequent experiment, the binding of purified NF-κB to a radiolabeled probe containing the frequently found NF-κB-binding site GG GACTTTCC (Fig. 1C) was inhibited by 2.5-, 25-, and 250-fold molar excesses of unlabeled oligonucleotides. This allowed us to estimate the relative affinities of NF-κB for the GM-CSF κB sites in comparison to well-characterized functional sites. The unlabeled oligonucleotide κB was the best competitor, indicating that NF-κB had the highest affinity for this site (Fig. 2B). The GM-CSF1 oligonucleotide inhibited the binding of NF-κB to the κB site better than the IL-2 oligonucleotide (Fig. 2B) and somewhat less than the IL-2R oligonucleotide (data not shown). An oligonucleotide containing the biologically active NF-κB-binding site from the beta interferon promoter (15, 28) showed a profile of competition similar to that of the GM-CSF1 oligonucleotide, indicating that NF-κB had a similar affinity for the two sites (Fig. 2B). An unrelated oligonucleotide caused no significant

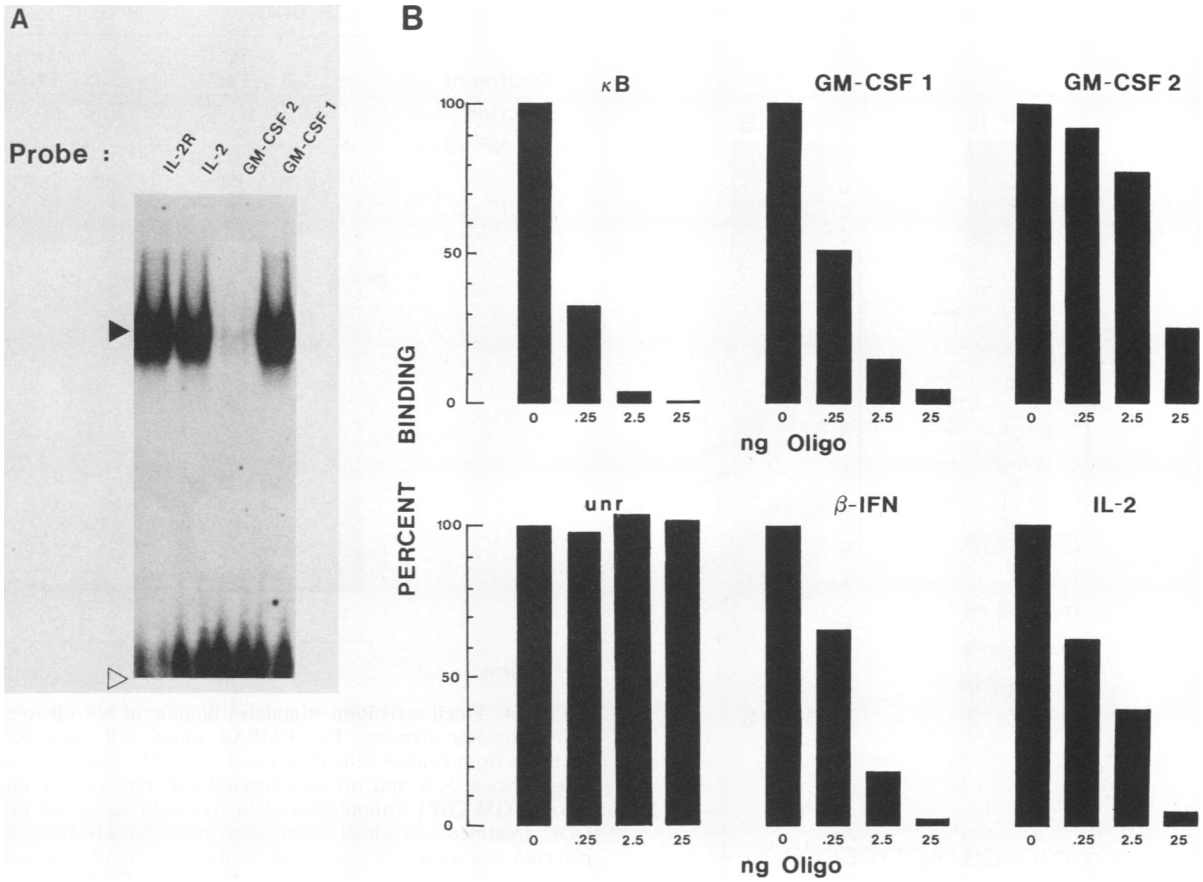

FIG. 2. Binding of purified NF-κB to sequences in the GM-CSF promoter in comparison to various other sites. (A) Direct binding analysis by EMSA. NF-κB (about 50 pg) purified from the cytosol of human placenta was reacted with various ^{32}P-labeled oligonucleotides and analyzed by EMSA. The sequences of the oligonucleotides corresponding to elements of the GM-CSF promoter are depicted in Fig. 1A. They were extended by *Sal*I and *Hin*dIII linker sites to 26-mers. For use in EMSAs, 50 ng of the annealed 34-mers was labeled with [α-^{32}P]dCTP (Amersham Corp.) with the Klenow enzyme (Boehringer GmbH). A fluorogram of a native gel is shown. Symbols: ▶, position of the NF-κB-DNA complex; ▷, position of free DNA. (B) Relative affinities of the NF-κB-binding sites. For binding reactions, six different unlabeled double-stranded oligonucleotides (0.25, 2.5, and 25 ng) were mixed with 0.1 ng of ^{32}P-end-labeled oligonucleotide containing the NF-κB-binding site of the mouse immunoglobulin κ light-chain enhancer (κB). Except for the nonspecific oligonucleotide (unr, unrelated; 40-mer), all others were 34-mers and annealed from 26-mers. The decameric NF-κB-binding site was located in the center and flanked by four to six nucleotides from the original *cis*-acting elements and by *Sal*I and *Hin*dIII linker sequences. The percentage of binding was calculated from the amount of the NF-κB-DNA complex in native gels (determined by Cerenkov counting), by comparison to the amount of complex obtained from binding reactions that did not contain cold competitor oligonucleotide. β-IFN, Beta interferon; Oligo, oligonucleotide.

competition of NF-κB binding within the concentration range tested. The order of affinities appeared to be as follows: κB > GM-CSF1 > beta interferon > IL-2. These in vitro data suggest that NF-κB can bind with high affinity to a site within the GM-CSF1 oligonucleotide. Because this oligonucleotide corresponds to a promoter element that confers inducible gene expression upon T-cell activation and upon tax_1 expression to a heterologous promoter, NF-κB appears to be the transcription factor responsible for mediating these effects.

As expected from the binding data shown in Fig. 2A, the GM-CSF2 oligonucleotide containing the other two potential NF-κB-binding sites in the GM-CSF promoter (Fig. 1C) competed only weakly with binding of NF-κB to the κB site (Fig. 2B). However, compared with an unrelated oligonucleotide, its competition was significant (Fig. 2B). Although NF-κB appears to bind with an affinity at least 1 order of magnitude lower than that of the site(s) in the GM-CSF2 oligonucleotide, its binding might still be relevant in vivo after activation of NF-κB by tax_1 and help to explain the weak tax_1 responsiveness localized to the CLE 1 motif (20).

Novel binding motif for NF-κB. A methylation interference analysis allowed us to determine how NF-κB contacted the high-affinity κB site in the mouse GM-CSF promoter. The method was performed as described previously (25), with the following modifications. After separation of DNA complexed by purified NF-κB from free DNA, the 4% polyacrylamide gel was immersed in EMSA running buffer (25) containing 0.2% sodium dodecyl sulfate. In the same buffer, the DNA was transferred from the gel onto DEAE paper by semidry blotting (Sartorius equipment) for 15 min at a constant current of 0.5 A. After 1 h of autoradiography, the portions containing the DNA were excised from the filter and eluted overnight at 65°C in TE–1 M NaCl–0.2% sodium dodecyl sulfate. The eluates were centrifuged and extracted with phenol-chloroform before cleavage by piperidine, as described previously (25).

The methylation of six purines in the noncoding strand and

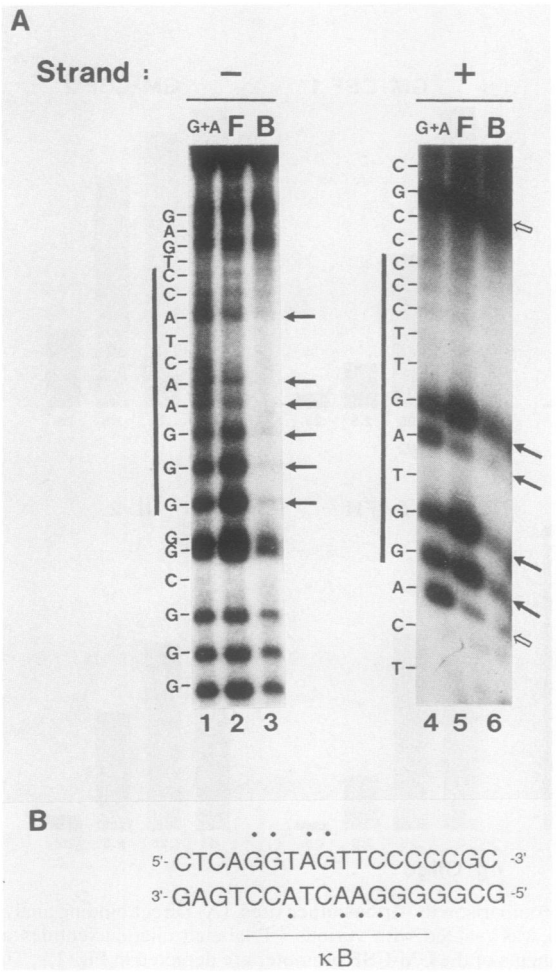

FIG. 3. Methylation interference analysis of a NF-κB–GM-CSF oligonucleotide complex. (A) Fluorogram of a 12% denaturing polyacrylamide gel. Lanes 1 and 4, purines (G+A); lanes 2 and 5, free DNA (F); lanes 3 and 6, complexed DNA (B). Symbols: ◀, Purine residues that interfere with binding of NF-κB when methylated; ◁, purines that do not interfere significantly with DNA binding of NF-κB when methylated. For the analysis, an oligonucleotide that contained GM-CSF sequences from −97 to −70 was used. (B) The coding (top) and noncoding (bottom) strands are shown. Symbol: ••••, purine residues essential for NF-κB binding.

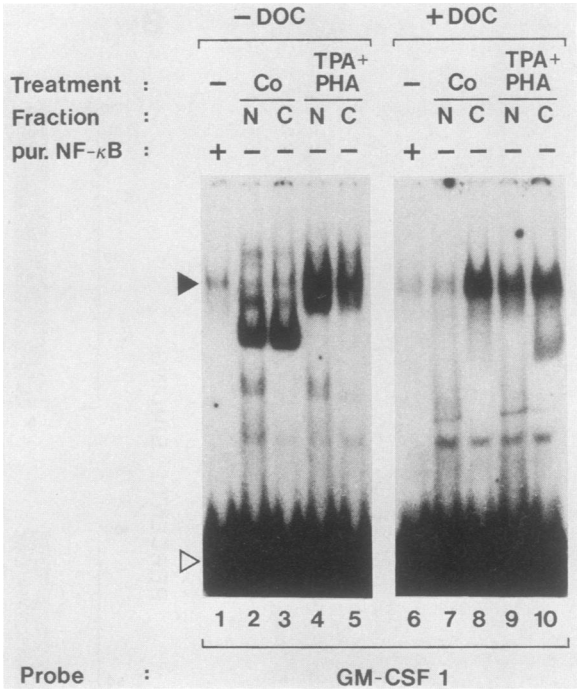

FIG. 4. T-cell activation stimulates binding of NF-κB to a GM-CSF promoter element. For EMSAs, equal cell equivalents of fractions from control cells (lanes 2, 3, 7, and 8) and from induced cells (lanes 4, 5, 9, and 10) were reacted with radiolabeled oligonucleotide GM-CSF1 without (lanes 1 to 5) or with (lanes 6 to 10) prior DOC treatment. A small amount of pure NF-κB from human placenta was used as a size standard in lanes 1 and 6. Fluorograms of native gels are shown. Symbols: ▶, NF-κB-DNA complex; ▷, free DNA probe. N, Nuclear fraction; C, cytosolic fraction; Co, control.

four purines in the coding strand interfered strongly with the binding of NF-κB (Fig. 3A). A 10-base-pair sequence at positions −82 to −91 of the GM-CSF promoter (Fig. 3B) could be defined as a binding site for NF-κB. It is very similar to other well-characterized NF-κB-binding sites, except for the adenosine in position 8 (Fig. 1C). The κB-like motifs at positions −108 to −98 were not amenable to methylation interference analysis, because only small amounts of complex were formed with NF-κB.

NF-κB activated in T cells by TPA-PHA treatment in vivo or by DOC treatment in vitro can bind to the GM-CSF promoter. We investigated whether the factor that is activated in the Jurkat human T-cell line upon treatment with T-cell-activating agents would bind to the GM-CSF1 oligonucleotide containing the high-affinity NF-κB-binding site. Jurkat T cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (both from GIBCO Laboratories). Stimulation with 5 μg of PHA and 50 ng of TPA (both from Sigma) per ml was performed for 8 h. A corresponding amount of dimethyl sulfoxide, the solvent for TPA, was added to control cultures. Cells were harvested by centrifugation and washed in phosphate-buffered saline at 4°C, and fractions were prepared as described by Dignam et al. (9) with the modifications described previously (1). Nuclear extracts and cytosolic fractions were kept frozen at −70°C. For the analysis of the subcellular fractions from Jurkat cells, equal cell equivalents (1.5 to 4 mg of protein per ml) were used in EMSAs. Treatment with DOC (E. Merck AG) was performed by adding 2.5 μl of 4% DOC to 2 μl of the fractions and then adding the DNA-binding mix and 2 μl of 10% (vol/vol) Nonidet P-40. Binding reactions were allowed for 30 min and were analyzed on native 4% polyacrylamide gels.

Under control conditions, the GM-CSF1 probe detected in EMSAs several DNA-binding activities in nuclear and cytosolic fractions, among them a very weak activity comigrating with the complex formed with purified human NF-κB (Fig. 4, lanes 1 to 3). Upon an 8-h treatment of Jurkat cells with PHA and TPA, a high increase of the DNA-binding activity comigrating with that of purified NF-κB was seen with the nuclear extract and to a small extent with the cytosolic fraction (Fig. 4, lanes 4 and 5). This activity was specifically inhibited by an unlabeled oligonucleotide containing the κB motif of the mouse κ light-chain enhancer (data not shown). To further support the idea that the activity detected by the GM-CSF1 probe in fractions from activated T cells was related to NF-κB, we analyzed nuclear and cytosolic fractions from control and stimulated cells after treatment with

DOC. This agent was shown to release NF-κB from a specific cytoplasmic inhibitory protein, IκB, that appears to be responsible for the inactivation and cytoplasmic localization of the NF-κB transcription factor in uninduced cells (2). The GM-CSF1 probe could indeed detect a form of NF-κB that was activated by DOC in cytosolic fractions (Fig. 4, lanes 8 and 10). This cytosolic NF-κB activity from T cells comigrated with that of NF-κB purified from human placenta (Fig. 4, lanes 6 to 10) and from HeLa cells (data not shown). T-cell activation led to a reduction of the DOC-dependent cytosolic activity and a concomitant increase of an active nuclear form of NF-κB (Fig. 4; compare lanes 7 and 8 with lanes 9 and 10). About 30% of the total cellular NF-κB was activated (Fig. 4; compare lanes 9 and 10). Interestingly, a faster-migrating complex seen under control conditions (Fig. 4, lanes 2 and 3) disappeared upon T-cell activation (lanes 4 and 5). We suspect that a constitutive factor binding to or overlapping the NF-κB site (like KBF1-H2TF1) became displaced upon binding of the inducible factor. Our observations strongly suggest that a DNA-binding activity indistinguishable from that of NF-κB is activated upon stimulation of human T cells by PHA and TPA. Like the purified NF-κB from human placenta, the NF-κB from the Jurkat T-cell line can bind to a κB motif in the GM-CSF gene and is thus likely to be the transcription factor conferring T-cell-activating signals to the GM-CSF promoter.

On the basis of all available criteria, this study demonstrates that the NF-κB transcription factor can bind to a key regulatory element in the GM-CSF promoter. (i) In EMSAs, purified human NF-κB can strongly complex a radiolabeled oligonucleotide corresponding to a previously characterized *cis*-acting element of the GM-CSF promoter. (ii) The unlabeled oligonucleotide is a strong competitor of binding of NF-κB to the κB motif GGGACTTTCC, comparable to other oligonucleotides containing well-characterized *cis*-acting κB motifs. (iii) The factor that is activated in human T cells upon TPA-PHA treatment and in cytosolic fractions by DOC is indistinguishable from NF-κB and apparently recognizes the κB motif in the GM-CSF promoter. The idea that an NF-κB-binding site is involved in the regulation of the GM-CSF gene is strongly supported by the mutation analysis data from Miyatake et al. (20). CAT constructs in which the NF-κB binding site at positions −82 to −91 was abolished by either removing sequences downstream of position −84 or deleting bases between positions −86 and −91 lost their inducibility upon treatment of cells with TPA-calcium ionophore and were reduced significantly in their responses to transient tax_1 expression. Further support comes from the analysis of the simian virus 40 enhancer (20). Only the segment containing the NF-κB-binding site could confer T-cell-activating signals and tax_1 expression in CAT assays. Consistent with an involvement of the NF-κB transcription factor in the inducible expression of the GM-CSF gene is the finding that the expression of the GM-CSF gene is induced upon treatment of cells with IL-1 (6) and tumor necrosis factor α (21); both cytokines were recently shown to activate NF-κB (10, 18, 24).

The weak responsiveness of the CLE 1 element to transient expression of tax_1 but not to T-cell-activating signals (20) might also be caused by the actions of NF-κB. As suggested by our in vitro binding and competition data, NF-κB can bind with low affinity to this element. If transient tax_1 expression in T cells converts more of the cytoplasmic NF-κB into its active form than the TPA-calcium ionophore treatment does, the low-affinity site in the GM-CSF promoter would preferentially respond to tax_1, the agent generating higher concentrations of active NF-κB. Because (i) the CLE 1 motif (also referred to as the CK-1 motif) is highly conserved among cytokine genes and (ii) NF-κB had only a weak affinity for this element, it is likely that a more specific factor with a DNA-binding specificity related to that of NF-κB recognizes this site. Such a factor might be $NF\text{-}GM_a$ (26).

From previous studies, it was unclear whether NF-κB or so-called NF-κB-like factors are activated in T cells upon stimulation with TPA-PHA (4, 5). The uncertainty came from the observation in EMSA of multiple complexes, the detection of several protein species in UV-cross-linking experiments, and DNA affinity precipitation experiments. Under our experimental conditions, we could not obtain any evidence that the factor activated in T cells is distinct from NF-κB. The protein-DNA complex of the NF-κB induced in Jurkat T cells after TPA-PHA treatment and of the NF-κB activated in vitro by DOC comigrated in native gels with the protein-DNA complex of human NF-κB purified from the cytosol of placenta and of HeLa cells. The NF-κB from HeLa cytosol was recently shown to be composed of two 50-kDa DNA-binding protein subunits and two 65-kDa non-DNA-binding protein subunits (2a). A 50-kDa protein was also evident from protein-DNA UV-cross-linking experiments with T-cell extracts (4, 8, 13). From the comigration of the complexes in native gels, we expect that the NF-κB in T cells has the same protein composition as the NF-κB from placenta and HeLa cells and is therefore a ubiquituous form of NF-κB. It is likely, however, that under certain assay conditions, the NF-κB heterotetramer is associated with other proteins, giving rise to more slowly migrating complexes. Alternatively, certain κB sites like the one in the IL-2R promoter may contain flanking sequences that allow additional proteins to bind to the DNA probe, thereby creating larger complexes.

The GM-CSF gene is a novel cytokine gene whose expression is controlled by binding of the NF-κB transcription factor. Other examples are the genes encoding IL-2 (13), tumor necrosis factor α (Shakov et al., J. Exp. Med., in press; M. Collart and P. Vassalli, Mol. Cell. Biol., in press), beta interferon (16, 28), and IL-6 (T. Lieberman and D. Baltimore, submitted for publication). A possible candidate is also the tumor necrosis factor β gene, for which we recently characterized a high-affinity binding site for NF-κB in the upsteam promoter region (G. Messer, E. Weiss, and P. Baeuerle, unpublished observation). As supported further by our study, NF-κB serves in a great variety of genes within upstream promoter and enhancer elements as a transcription factor that can rapidly induce mRNA synthesis upon extracellular stimulation. In particular, within so-called cytokine networks (27), NF-κB appears to have a key role in signaling. Because several cytokines can activate NF-κB and because the active NF-κB in turn can upregulate the transcription of these and other cytokines, this transcription factor can mediate positive autoregulation of cytokine synthesis as well as cross-talking between distinct cytokine systems.

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