A Novel Tumor Necrosis Factor-Responsive Transcription Factor Which Recognizes a Regulatory Element in Hemopoietic Growth Factor Genes

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A conserved DNA sequence element, termed cytokine 1 (CK-1), is found in the promoter regions of many hemopoietic growth factor (HGF) genes. Mutational analyses and modification interference experiments show that this sequence specifically binds a nuclear transcription factor, NF-GMa, which is a protein with a molecular mass of 43 kilodaltons. It interacts with different affinities with the CK-1-like sequence from a number of HGF genes, including granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte (G)-CSF, interleukin 3 (IL-3), and IL-5. We show here that the level of NF-GMa binding is induced in embryonic fibroblasts by tumor necrosis factor-α (TNF-α) treatment and that the CK-1 sequence from the G-CSF gene is a TNF-α-responsive enhancer in these cells. The NF-GMa protein is distinct from another TNF-α-responsive transcription factor, NF-κB, by several criteria. Firstly, several NF-κB-binding sites, although having sequence similarity with the CK-1 sequence, cannot compete efficiently for NF-GMa binding to CK-1. Secondly, the CK-1 sequence from both G-CSF and GM-CSF does not respond to phorbol ester treatment as would an NF-κB-binding element. These results demonstrate that NF-GMa is a novel transcription factor inducible by TNF-α and binds to a common element in HGF gene promoters.

Hemopoietic growth factors (HGFs) are a family of glycoproteins involved in the stimulation of proliferation and differentiation of progenitor cells and in the activation of function of mature hemopoietic cells (reviewed in reference 28). Expression of the genes encoding these glycoproteins is tightly regulated, with little constitutive production observed. Expression of one or a subset of these genes can, however, be induced by a range of stimuli in different cell types (reviewed in reference 6). The relative level of expression from the genes can also be altered by different stimuli in the same cell type. Antigen or mitogen activation of T cells leads to the increased synthesis of several HGFs, including granulocyte-macrophage colony-stimulating factor (GM-CSF) (50), interleukin 3 (IL-3) (35, 52), and IL-5 (46). Monocytes and macrophages can produce GM-CSF, granulocyte (G)-CSF, and monocyte-CSF following treatment with agents such as lipopolysaccharide (9, 39, 47). Fibroblasts and endothelial cells can be stimulated by tumor necrosis factor-α (TNF-α) or IL-1 to produce GM-CSF, G-CSF, monocyte-CSF, and IL-6 (3, 4, 19, 21, 31, 53). In monocytes, increased mRNA stability may account for induced expression (9), whereas in fibroblasts and endothelial cells, both increased mRNA stability (22) and increased transcriptional activity of the genes (22, 40) are responsible for the increase in HGF expression.

The capacity to produce one or several of these proteins suggests that their transcription could be regulated either individually or as a group. There exist a number of conserved sequences in the promoter regions of many genes, including HGF genes, that are potential transcriptional regulatory sequences. One highly observed sequence which we have termed cytokine 1 (CK-1; 5'GRGR/TTTY/ACY/AN3',

in which R = purine, Y = pyrimidine, and N = any nucleotide) has been identified only in the promoter region of several hemopoietic growth-factor genes, including GM-CSF, G-CSF, IL-3, IL-5, IL-2, IL-4, and IL-6 (Table 1) (30, 43, 45). A second sequence, called CK-2 (5'TCAGGTA3'), immediately downstream of CK-1 is restricted to the GM-CSF and IL-3 genes (43). Deletional analyses have agreed that the CK-1 sequence of both the human and mouse GM-CSF promoters appears to have no role in either basal-level expression or in phorbol ester induction in some T-cell lines (31, 32, 37). On the other hand, the CK-2 sequence is required for phorbol ester inducibility of the mouse but not the human gene promoter (32, 37). Both the CK-1 and CK-2 sequences appear to play a role in mouse GM-CSF promoter activation by the transactivator of the human T-cell leukemia virus type 1 (p40ˣ) (15, 32). In contrast, sequences closer to the transcription start site are thought to be responsible for human GM-CSF promoter activation by p40ˣ and phorbol ester (36, 37). The role of these sequences in gene induction in other cell types has not been extensively investigated, but a recent report suggests that IL-1 induction of GM-CSF in endothelial cells is mediated by a sequence at −63 relative to the CAP site (18).

DNA fragments spanning the conserved CK-1 sequence from the human GM-CSF and G-CSF genes specifically bind a nuclear protein called NF-GMa, which has been found in a number of cell types (43). The NF-GMb protein, which is phorbol ester inducible in the 5637 bladder carcinoma cell line (43), requires the intact CK-2 region of the GM-CSF promoter for binding (44). The CK-1-like sequence in the IL-2 gene (Table 1) has recently been shown to bind the transcription factor NF-κB (23). NF-κB has been shown to mediate phorbol ester inducibility of many genes, including IL-2 (16). Also, NF-κB mediates the TNF-α induction of the

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TABLE 1. Conserved sequences in hemopoietic growth factor gene promoters

HGF gene[a]	Sequence[b]
hGM-CSF (1)	−96　　　　　　　　　　　　　　　　−77 G A G A T T C C A C A G T T C A G G T A
hGM-CSF (2)	−357　　　　　−346 G g G A T T a C A g
mGM-CSF	−108　　　　　　　　　　　　　　−89 G A G A T T C C A C A a c T C A G G T A
hIL-3 (1)	−126　　　　　　　　　　　−109 G A G g T T C C A t − g − T C A G a T A
hIL-3 (2)	−331　　　　　−322 G A G A T c C C A C
mIL-3 (1)	−115　　　　　　　　　　−98 G A G g T T C C A t − g − T g A G a T A
mIL-3 (2)	−198　　　　−189 G A G A T T C C A C
hIL-2	−208　　　　−199 G g G A T T t C A C
mIL-2	−208　　　　−199 G g G A T T t C A C
hG-CSF	−188　　　　−179 G A G A T T C C A C
mG-CSF	−192　　　　−181 G A G A T T C C c C
hIL-5	−223　　　　−214 a A G A T T C t t C
hIL-6	−136　　　　−127 a g G t T T C C A a
hIL-4 (R)	−178　　　　−169 G A a A T T a C A C

[a] The HGF gene names are abbreviated as in the text, with the addition of hIL-4 (human IL-4). h, Human; m, mouse; R, sequence in the reverse orientation relative to the direction of transcription. The numbers in parentheses, (1) and (2), refer to two copies of the sequence in the same promoter.

[b] The altered bases, when compared with the GM-CSF sequence, are shown in lowercase letters. The numbers above the sequence refer to the distance from the transcription start site.

human immunodeficiency virus (HIV) enhancer and the IL-2 receptor α-chain gene (25, 38). Since genes containing the CK-1 sequence are also phorbol-12-myristate-13-acetate (PMA) and TNF inducible, the relationship between NF-GMa and NF-κB is of interest.

In the present study, we have characterized the transcription factor NF-GMa and suggest that it is involved in the control of HGF gene transcription. The NF-GMa protein has a molecular mass of 43 kilodaltons and binds with a range of affinities to the CK-1 sequence from several HGF genes. It is distinct from a second GM-CSF promoter-binding protein, NF-GMb, which we show binds to the adjacent CK-2 sequence. In primary embryonic fibroblasts, the level of NF-GMa transiently increases with TNF-α treatment and the G-CSF CK-1 sequence acts as a TNF-responsive enhancer in transfection experiments. Although there is sequence similarity between the binding sites for NF-GMa and NF-κB, these two proteins show high-affinity binding to distinct sets of genes. Transfection experiments have also shown that although the CK-1 sequence is a TNF-α-inducible enhancer, unlike NF-κB it does not respond to phorbol ester.

MATERIALS AND METHODS

Oligonucleotides and plasmids. All oligonucleotides were synthesized on an Applied Biosystems model 381A DNA synthesizer, and full-length material was purified by polyacrylamide gel electrophoresis. The sequences of the oligonucleotides are described for individual experiments. Following treatment with kinase and annealing (27), double-stranded oligonucleotides were cloned into the Bluescript SK(−) vector (Stratagene Inc.) by using EcoRI or SmaI cloning sites. Clones containing single or multiple copies of the oligonucleotides were selected, and the sequences and orientations were checked by double-stranded sequencing (5). pSKCK-1/2 and pSKCK-1 contained a single copy of the GM-CSF and G-CSF oligonucleotides, respectively (Fig. 1A), and were used to generate probes for gel retardation, modification interference, and UV cross-linking. Single or multiple inserts of the G-CSF or multiple inserts of the GM-CSF oligonucleotides (Fig. 1A) were excised from Bluescript clones by using BamHI-HindIII and were directionally cloned into pBLCAT2 (26) upstream of the thymidine kinase (tk) promoter and the chloramphenicol acetyltransferase (CAT) reporter gene to generate the following constructs for transfection. pCK-1(4+) and pCK-1(4−) contained four copies of the G-CSF CK-1 sequence, and pCK-1/2(5+) and pCK-1/2(5−) contained five copies of the GM-CSF CK-1-CK-2 sequence (see Fig. 6A), with plus and minus indicating opposite orientations. To generate pM1(3) and pM4(3), three copies of the M1 and M4 mutant oligonucleotides, respectively, were cloned upstream of the tk promoter as described above. The IL-2 probe was as previously described (23).

Cell lines. 5637 is a human bladder carcinoma cell line that constitutively produces low levels of GM-CSF and G-CSF. Treatment with PMA increases the levels of GM-CSF and G-CSF mRNA and protein (49). HUT78 is a T-lymphoblastoid cell line (12) which synthesizes low levels of GM-CSF and responds to PMA with increased synthesis of GM-CSF mRNA (unpublished observations). Primary embryonic fibroblasts were obtained from Flow Laboratories, Inc., and used at passages 16 to 25 in all experiments. All cell lines were routinely grown in RPMI medium supplemented with
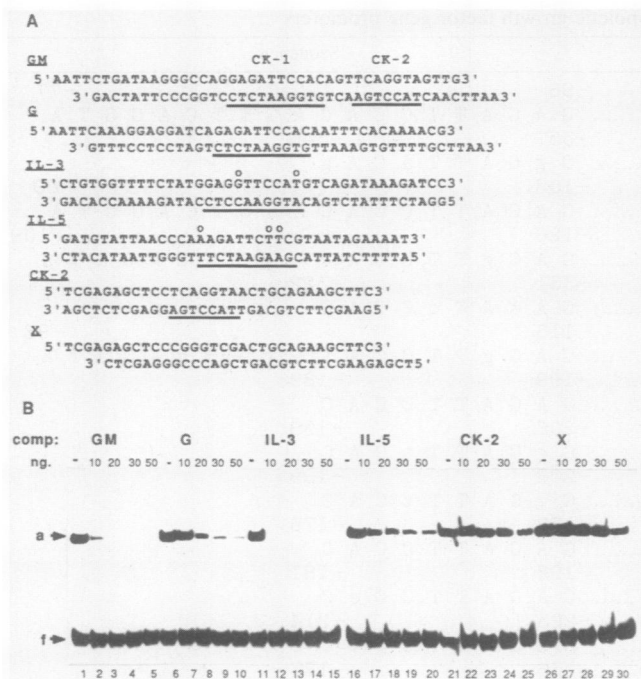
A

GM
```
5'AATTCTGATAAGGGCCAGGAGATTCCACAGTTCAGGTAGTTG3'
   3'GACTATTCCCGGTCCTCTAAGGTGTCAAGTCCATCAACTTAA3'
```
G
```
5'AATTCAAAGGAGGATCAGAGATTCCACAATTTCACAAAACG3'
   3'GTTTCCTCCTAGTCTCTAAGGTGTTAAAGTGTTTTGCTTAA3'
```
IL-3
```
5'CTGTGGTTTTCTATGGAGGTTCCATGTCAGATAAAGATCC3'
   3'GACACCAAAAGATACCTCCAAGGTACAGTCTATTTCTAGG5'
```
IL-5
```
5'GATGTATTAACCCAAAGATTCTTCGTAATAGAAAAT3'
   3'CTACATAATTGGGTTTCTAAGAAGCATTATCTTTTA5'
```
CK-2
```
5'TCGAGAGCTCCTCAGGTAACTGCAGAAGCTTC3'
   3'AGCTCTCGAGGAGTCCATTGACGTCTTCGAAG5'
```
X
```
5'TCGAGAGCTCCCGGGTCGACTGCAGAAGCTTC3'
   3'CTCGAGGGCCCAGCTGACGTCTTCGAAGAGCT5'
```

B

comp: GM   G   IL-3   IL-5   CK-2   X
ng. - 10 20 30 50 - 10 20 30 50 - 10 20 30 50 - 10 20 30 50 - 10 20 30 50 - 10 20 30 50

a →

f →

     1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

FIG. 1. NF-GMa binding to the CK-1 sequence from several HGF genes. (A) Sequences of the double-stranded synthetic oligonucleotides spanning the CK-1-CK-2 sequences of the GM-CSF, G-CSF, IL-3, and IL-5 genes which were used in competition experiments. The CK-2 sequence from GM-CSF was placed in a random DNA sequence to generate the CK-2 oligonucleotide. X is a random polylinker DNA fragment used as a nonspecific competitor. The CK-1 and CK-2 regions are underlined, and the base changes within these regions compared with the GM-CSF sequence are indicated (O). (B) Gel retardation binding assay using 1 μg of heparin-Sepharose-enriched NF-GMa from HUT78 T cells and radiolabeled GM sequence as a probe (0.2 ng). Binding reactions contained either no competitor (lanes 1, 6, 11, 16, 21, and 26) or GM (lanes 2 through 5), G (lanes 7 through 11), IL-3 (lanes 12 through 15), IL-5 (lanes 17 through 20), CK-2 (lanes 22 through 25), or X (lanes 27 through 30) in the increasing nanogram amounts shown for each lane. The NF-GMa complex is indicated by the letter a and the unbound DNA by the letter f.

10% fetal calf serum. Cells were harvested for extract preparation at 10^6 cells per ml or at 80% confluence for nonadherent and adherent cell lines, respectively. TNF-α (100 U/ml) was added to the fibroblast cells in fresh medium, and cells were harvested for nuclear extract preparation at different times following treatment.

Preparation of nuclear extracts and gel retardation assays. Nuclei were prepared from all cell types as described by Dignam et al. (8). Nuclear proteins were prepared as previously described (43), except that extraction of the nuclei with 0.5 M KCl was for 60 min at 4°C. The protein extracts were stored at −70°C in TM.1 (50 mM Tris hydrochloride [pH 7.6], 12 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, 100 mM KCl) (17). Protein concentration was determined by the method of Bradford (2). Purified NF-κB was prepared as described by Lenardo et al. (23).

Probes for gel retardation were prepared as previously described (43) or by end labeling of restriction fragments from pSKCK-1 or pSKCK-1/2 plasmids. Competitor DNAs were prepared by annealing the appropriate synthetic oligonucleotide pairs in 25 mM Tris hydrochloride (pH 7.6)–150 mM KCl at a final concentration of 10 ng/μl. For NF-

GMa-binding reactions, 0.1 to 0.5 ng of radiolabeled double-stranded oligonucleotides or restriction fragment (5,000 to 10,000 cpm) was mixed with 1 to 3 μg of crude nuclear extract or the indicated amount of enriched or purified material in a final volume of 20 μl containing 25 mM Tris hydrochloride, pH 7.6, 6.25 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 200 mM KCl. Poly(dI:dC) (0.5 to 2.0 μg) was used as nonspecific competitor in the reactions. For binding competition experiments, specific competitor DNAs were always mixed with the reaction before addition of the radiolabeled probe. The reactions were analyzed on 12% polyacrylamide gels in 0.5× TBE (43). NF-κB binding was detected as previously described (23).

UV cross-linking. For UV cross-linking experiments, probes were generated by primed synthesis on collapsed double-stranded DNA (5) by using 50 μM 5-bromodeoxyuridine and [α-^{32}P]dATP and by using pSKCK-1/2. The DNA was then cleaved with EcoRI, and the 40-base-pair (bp) insert was purified on polyacrylamide gels. Binding reactions for UV cross-linking were carried out as described above, but in a final volume of 50 μl, by using 6 to 10 μg of nuclear extract and 1 ng of radiolabeled probe containing 5-bromodeoxyuridine. Following UV irradiation (340 nm) for 15 min, complexes were separated on the standard gel retardation system. The wet gel was exposed to X-ray film for 6 to 12 h, and the area of the gel containing the retarded band was excised and incubated in sodium dodecyl sulfate load buffer for 30 min at 37°C. The gel slice was placed directly in the well of a 10% Laemmli protein gel, overlaid with 1% agarose, and electrophoresed at 30 mA for 6 h. The gel was stained with Coomassie blue and destained to visualize the markers, dried, and exposed to X-ray film for 1 to 3 days.

Modification interference. Modification interference reactions were carried out by the method of Gilman et al. (13), except that both A and G residues were modified with formic acid for 5 to 10 min prior to the binding reactions. Concentrated protein fractions, from heparin-Sepharose chromatography of crude nuclear extract (43), containing either NF-GMa or NF-GMb were used. Probes were generated by end labeling either the HindIII or BamHI sites in pSKCK-1 or pSKCK-1/2 and excising the labeled fragment with the opposite enzyme. The modified end-labeled fragments were used in scaled-up gel retardation assays with either 10 or 5 μg of protein enriched for NF-GMa or NF-GMb, respectively.

Transfection of cells. Both fibroblasts and 5637 cells were plated at a density of 1×10^6 to 1.5×10^6 cells per 100-mm dish and grown for 2 to 3 days. Plasmid DNA (10 μg per dish) was transfected by using DEAE dextran (400 μg/ml for 2 h) and a 10% dimethyl sulfoxide shock (41). Cells were treated 24 h later with TNF-α (100 U/ml) or PMA (20 ng/ml) for 12 to 16 h. Cytoplasmic extracts were prepared, and CAT assays were carried out by using 25 to 50 μg of cell extract per assay (14). Percent CAT conversion was determined by scintillation counting of thin-layer chromatography plate areas containing [^{14}C]chloramphenicol and its acetylated derivatives. Relative CAT activity was determined within each experiment from the value obtained for pBLCAT2.

RNA analysis. Total cytoplasmic RNA was prepared from 5637 cells that had either been treated with PMA for 6 h or left untreated. Northern (RNA) blots (15 μg per track of RNA) were probed with a 600-bp insert from pSP6GM-CSF cDNA clone (20) labeled by random priming (10).
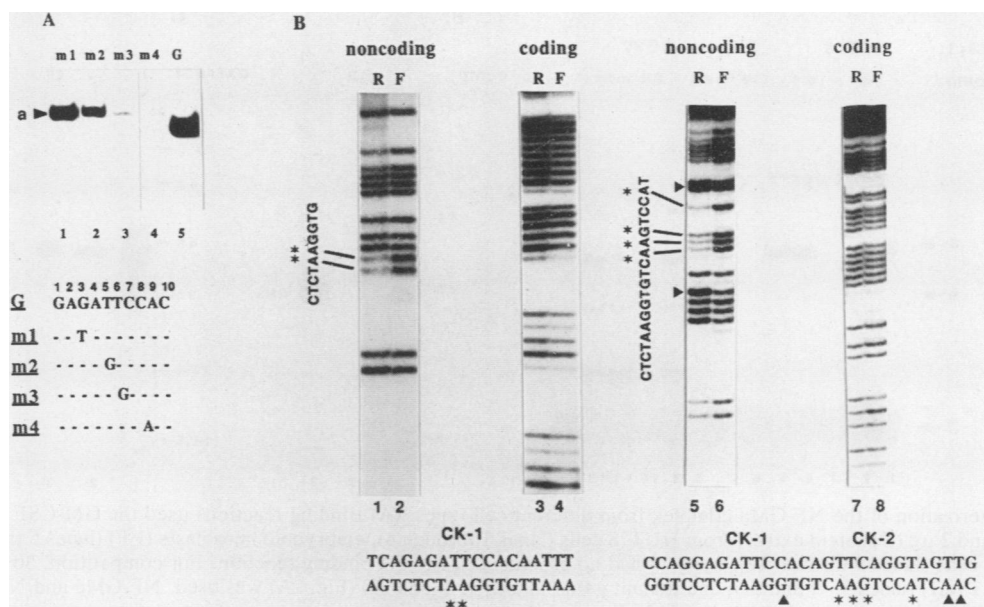
FIG. 2. Binding requirements for NF-GMa and NF-GMb. (A) Single-point mutations were generated within the CK-1 sequence of the G-CSF gene, and each mutant was tested for its ability to generate the NF-GMa complex from heparin-Sepharose-enriched HUT78 cell extract. Only the retarded complex NF-GMa is shown. Lane 1, Mutant 1 (m1); lane 2, mutant 2 (m2); lane 3, mutant 3 (m3); lane 4, mutant 4 (m4); lane 5, G-CSF CK-1 sequence. The complexes formed with mutant sequences migrate more slowly on the gel because they are all 30 bp long compared with 40 bp for the G-CSF sequence. The single-point mutations are shown under the G-CSF CK-1 sequence. Flanking sequences were maintained as in the G-CSF flanking sequence. (B) Modification interference experiments with NF-GMa-enriched (lanes 1 through 4) and NF-GMb-enriched HUT78 extract (lanes 5 through 8). Free DNA (F) and retarded DNA protein complex (R) were eluted from a scaled-up retardation gel, and the piperidine cleavage pattern was analyzed on a 6% denaturing polyacrylamide gel. A 70-bp DNA fragment containing the G-CSF CK-1 sequence from pSKCK-1 (see Materials and Methods) (lanes 1 through 4) or the GM-CSF CK-1/2 sequence excised from pSKCK-1/2 (lanes 5 through 8) was end labeled on either the noncoding (lanes 1, 2, 5, and 6) or coding strands (lanes 3, 4, 7, and 8) and used as probes in the binding assays. The modified bases which interfered with (*) or enhanced (▲) NF-GMa or NF-GMb binding are indicated. The positions of the CK-1 and CK-2 sequences are shown.

RESULTS

NF-GMa binds to several CK-1-containing DNA fragments. We have previously shown that two DNA-protein complexes, NF-GMa and NF-GMb, are formed with crude nuclear extract from PMA-stimulated 5637 cells binding to a short DNA fragment spanning the highly conserved CK-1 (5'GAGATTCCAC3') and CK-2 (5'TCAGTCA3') sequences of the human GM-CSF promoter (43). Only the NF-GMa complex is formed when a fragment of DNA from the G-CSF promoter containing CK-1 but not CK-2 is used as a probe in gel retardation assays (44). Double-stranded oligonucleotides spanning the CK-1 sequence in GM-CSF, G-CSF, IL-3, and IL-5 (Fig. 1A) surrounded by their naturally occurring flanking sequences were tested on retardation gels for their ability to compete for NF-GMa binding to a radiolabeled GM-CSF CK-1 sequence. NF-GMa enriched by heparin-Sepharose chromatography was used in all binding studies (43). Each CK-1 sequence was found to compete for NF-GMa binding to GM-CSF but with varying affinities (Fig. 1B). The relative affinity of each sequence, as determined by densitometer scanning of the gels, was IL-3 > GM-CSF > G-CSF > IL-5. The CK-2 sequence from GM-CSF placed in a random altered context and an unrelated piece of polylinker DNA did not significantly compete for NF-GMa binding (Fig. 1B). These results imply that CK-1 is the binding site for NF-GMa.

CK-1 is the binding site for NF-GMa. To define more precisely the binding requirements for NF-GMa, we introduced single-base mutations into the most highly conserved bases in the CK-1 sequence from G-CSF (Fig. 2A) and examined the ability of these mutant CK-1 sequences to bind NF-GMa. Each mutation reduced, to some degree, the ability of the sequence to bind NF-GMa. Mutation of either the T residue at position 6 to a G or the C residue at position 8 to an A reduced the binding affinity by >90% (Fig. 2A). Altering residue 5 (T to G) or residue 3 (G to T) also inhibited binding by 60 and 50%, respectively (Fig. 2A). The ability of these mutant CK-1 sequences to compete for binding of NF-GMa to the G-CSF CK-1 sequence parallels their ability to bind NF-GMa (data not shown). Single-base alterations have, therefore, defined several important nucleotides for NF-GMa binding to CK-1.

In order to further define the contact points of NF-GMa with the DNA, modification interference experiments were carried out. Modification of the central two A residues on the noncoding strand of the CK-1 sequence yielded a reproducible, albeit incomplete, interference pattern (Fig. 2B). Both of these bases were previously shown to affect binding when mutated (Fig. 2A).

CK-2 is the binding site for NF-GMb. Mutations within the CK-2 sequence have been shown to abolish NF-GMb but not NF-GMa binding to the GM-CSF sequence (44). Modification interference experiments were carried out with heparin-Sepharose-enriched NF-GMb (43). The interference pattern generated with NF-GMb was centered over the CK-2 sequence on the coding strand of the DNA fragment (Fig. 2B). Depurination at four bases within the sequence interfered with binding, whereas modification of some flanking bases
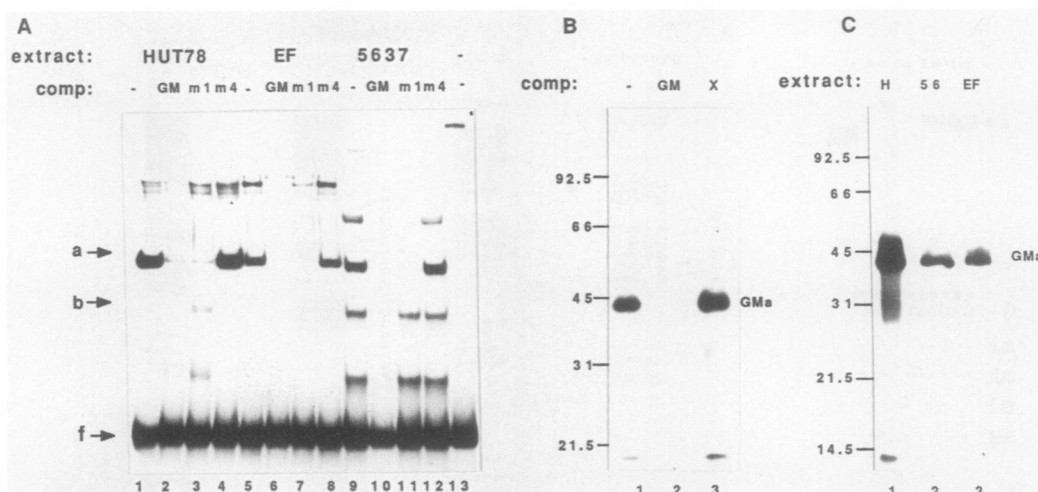
FIG. 3. Characterization of the NF-GMa complex from different cell types. (A) Binding reactions used the GM-CSF radiolabeled probe (0.2 ng) (Fig. 1A) and 2 μg of protein extract from HUT78 cells (lanes 1 through 4), embryonic fibroblasts (EF) (lanes 5 through 8), and 5637 bladder carcinoma cells (lanes 9 through 12). Lane 13 had no protein added to the binding reaction. For competition, 30 ng of GM (lanes 2, 6, and 10), mutant 1 (m1) (lanes 3, 7, and 11), and mutant 4 (m4) (lanes 4, 8, and 12) (Fig. 2A) was used. NF-GMa and NF-GMb are labeled a and b respectively, and f is unretarded DNA. (B) UV cross-linking experiments to determine the protein component(s) of the NF-GMa complex. Binding reactions contained 1 ng of 5-bromodeoxyuridine-labeled GM-CSF probe from pSKCK-1/2 and 5 μg of HUT78 extract. The cross-linked complexes were excised from a standard retardation gel and analyzed on a 12% sodium dodecyl sulfate-polyacrylamide gel. Binding reactions contained no competitor (lane 1), 50 ng of GM oligonucleotide (lane 2), or 50 ng of X oligonucleotide (lane 3). The positions and sizes of molecular weight markers are shown. (C) Sodium dodecyl sulfate-polyacrylamide gel of UV cross-linked NF-GMa complex from HUT78 cells (H, lane 1), 5637 cells (56, lane 2) and embryonic fibroblasts (EF, lane 3). Binding conditions were as described above.

appeared to enhance binding (Fig. 2B). These results show that the two proteins, NF-GMa and NF-GMb, form distinct complexes with adjacent DNA sequences.

Characterization of the protein(s) involved in the NF-GMa complex. We have previously detected NF-GMa binding in several cell types, including the bladder carcinoma cell line 5637, the lymphoblastoid cell line HUT78, and the melanoma cell line LiBr (43). The NF-GMa complex from the previously characterized 5637 and HUT78 cells and also from primary embryonic fibroblasts, in which low levels of NF-GMa can be detected, behaved in an identical manner in binding competition experiments with both wild-type and mutant CK-1 sequences (Fig. 3A). The identical behavior of the NF-GMa complex from different cell lines suggested that the same binding site and the same protein(s) are involved in the NF-GMa complex in each cell line.

To identify the molecular weight of the protein(s) responsible for forming the NF-GMa complex, a modification of the UV cross-linking method of Wu et al. (51) was used. A 40-bp fragment of DNA containing the G-CSF CK-1 sequence, labeled with [^{32}P]dATP and 5-bromodeoxyuridine, was used as a probe in a scaled-up binding reaction. Following exposure to UV light, the NF-GMa complex was separated on a standard polyacrylamide retardation gel. The NF-GMa complex was excised and reelectrophoresed on a 12% sodium dodecyl sulfate-polyacrylamide gel. A major protein band with a molecular mass of 43 kDa was identified on the protein gel (Fig. 3B). When excess unlabeled CK-1 competitor was added to the binding reaction, the 43-kDa protein band was eliminated, but a similar excess of an unrelated DNA fragment had no effect (Fig. 3B). In addition, complexes formed from extracts of HUT78, 5637, and fibroblast cells contained a single protein band of the same molecular weight, as determined by UV cross-linking (Fig. 3C). These results indicate that the NF-GMa complex involves at least one binding protein of 43 kDa.

NF-GMa and NF-κB have distinct binding requirements. The CK-1-like sequence from the IL-2 gene (11) has previously been regarded as a potential NF-GMa-binding site (43). Recently, however, this IL-2 sequence has been shown to bind with high affinity the transcription factor NF-κB, (16, 23). The IL-2 NF-κB-binding site differs from the GM-CSF CK-1 sequence by only two bases (Table 1). In order to determine whether NF-κB-binding sites can compete for binding of NF-GMa to the CK-1 sequence, competition experiments were carried out with increasing concentrations of unlabeled GM-CSF CK-1 sites or NF-κB-binding sites from the HIV enhancer (34), the IL-2 gene promoter (23), or the H2k^b gene H2TF1-binding site (1) (Fig. 4A). Although the homologous CK-1 sequence abolished the binding at 50-fold molar excess of cold DNA, none of the NF-κB-binding sites competed efficiently for NF-GMa binding (Fig. 4A). At high concentrations (>30 ng), the H2TF1-binding site showed a low level of competition (Fig. 4A).

We also tested the ability of purified NF-κB protein (23) to bind to the CK-1 sequence. Specific binding of NF-κB was detected to the IL-2 NF-κB site, but no retarded complexes were formed with the CK-1 sequence from either GM-CSF or G-CSF (Fig. 4B). Purified NF-GMa, on the other hand, bound with very low affinity to the NF-κB site from both the HIV enhancer and the IL-2 gene (data not shown). These data show that NF-GMa is distinct from NF-κB and that the IL-2 site has different binding properties to other CK-1-like sequences in hemopoietic growth factor genes.

The G-CSF CK-1 sequence acts as a TNF-α-responsive element in fibroblasts. Since several HGF genes which contain a CK-1-like sequence are TNF-α inducible in fibroblasts, we examined the possibility that NF-GMa and its binding site might play a role in this induction. Nuclear extracts prepared from primary human embryonic lung fibroblasts contained low levels of NF-GMa-binding activity compared with that previously seen in a number of perma-
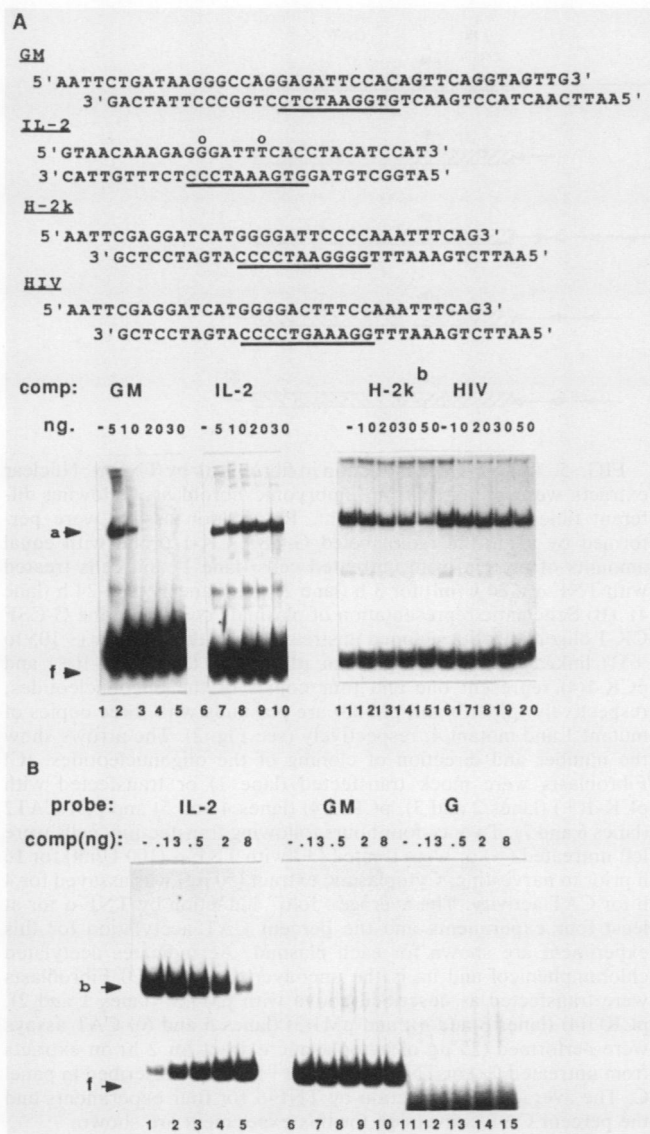
A

GM
5'AATTCTGATAAGGGCCAGGAGATTCCACAGTTCAGGTAGTTG3'
     3'GACTATTCCCGGTCCTCTAAGGTGTCAAGTCCATCAACTTAA5'

IL-2
5'GTAACAAAGAGGGATTTCACCTACATCCAT3'
3'CATTGTTTCTCCCTAAAGTGGATGTCGGTA5'

H-2k
5'AATTCGAGGATCATGGGGATTCCCCAAATTTCAG3'
    3'GCTCCTAGTACCCCTAAGGGGTTTAAAGTCTTAA5'

HIV
5'AATTCGAGGATCATGGGGACTTTCCAAATTTCAG3'
    3'GCTCCTAGTACCCCTGAAAGGTTTAAAGTCTTAA5'

comp: GM   IL-2   H-2k b   HIV

ng.   - 5 10 20 30   - 5 10 20 30   - 10 20 30 50 - 10 20 30 50

a ▶

f ▶

1 2   3 4 5   6 7 8 9 10   11 12 13 14 15 16 17 18 19 20

B

probe: IL-2   GM   G

comp(ng):   - .1 3 .5 2 8   - .1 3 .5 2 8   - .1 3 .5 2 8

b ▶

f ▶

1 2 3 4 5   6 7 8 9 10 11 12 13 14 15

FIG. 4. NF-GMa and NF-κB have distinct binding sites. (A) Sequences of oligonucleotides from the GM-CSF, IL-2, H-2k[b] genes, and the HIV enhancer containing either CK-1 (GM) or NF-κB-binding sites (IL-2, H-2k[b], and HIV), with the relevant sequences underlined. The base differences between the GM-CSF CK-1 and the IL-2 NF-κB sites are shown (○). Protein extract from HUT78 cells (2 μg) was used with the GM-CSF CK-1 sequence (0.2 ng) to generate the NF-GMa complex (a). Unlabeled competitor for GM (lanes 2 through 5), IL-2 (lanes 7 through 10), H-2k[b] (lanes 12 through 15), and HIV (lanes 17 through 20) was added to binding reactions in the nanogram amounts shown on the lanes. Lanes 1, 6, 11, and 16 had no competitor added. (B) Binding of purified NF-κB from bovine spleen to a DNA fragment containing the IL-2 NF-κB sequence (lanes 1 through 5), the GM-CSF CK-1/CK-2 sequence (lanes 6 through 10), or the G-CSF CK-1 sequence (lanes 11 through 15). Competitor IL-2 NF-κB sequence was added to binding reactions in the nanogram quantities indicated. Lanes 1, 6, and 11 had no competitor added. NF-κB complex (b) and the unbound DNA (f) are indicated.

nent cell lines in which GM-CSF and G-CSF could be induced (Fig. 5A). A time-dependent transient increase in the amount of NF-GMa was seen following treatment with TNF-α (100 U/ml) (Fig. 5A). Densitometric scanning of tracks from four independent experiments showed that the

maximum increase of fivefold was seen at 6 to 8 h of TNF-α treatment. By 24 h the amount of NF-GMa had returned to pretreatment levels (Fig. 5A). A similar time course of TNF induction of NF-GMa was observed in human umbilical vein endothelial cells (data not shown).

To determine whether the increased levels of NF-GMa described above are associated with increased transcriptional activity of the CK-1 sequence, single and multiple copies of the G-CSF CK-1 sequence were cloned upstream of the tk promoter and the CAT gene in the pBLCAT2 vector (26) (Fig. 5B). CAT activity was measured following transient transfection into fibroblasts. An increase of 12-fold (average of nine experiments) in CAT activity was observed following TNF-α treatment of cells transfected with a plasmid containing four copies of the CK-1 sequence [pCK-1(4+)] (Fig. 5C). A single copy of the CK-1 sequence [pCK-1(+)] gave low levels of CAT activity, but an average threefold increase in CAT levels was observed following TNF-α treatment. pM1(3) and pM4(3) (Fig. 5B), plasmids containing three copies of mutant 1 and mutant 4, respectively (Fig. 1A), were also transfected into fibroblasts and tested for their TNF responsiveness. Transcription from pM1(3) but not pM4(3) was induced approximately fourfold by TNF-α treatment (Fig. 5D), a result which corresponds with the ability of these mutants to bind NF-GMa (Fig. 1A). Cells transfected with the vector alone had undetectable CAT levels and did not respond to TNF-α treatment (Fig. 5C). The TNF-α response of the CK-1 sequence was both time- and dose-dependent, with maximum induction levels reached at 16 to 24 h and 100 U of TNF-α per ml (data not shown). These results show that the CK-1 sequence from G-CSF is a TNF-α-responsive element and that the response is probably mediated by increased levels of NF-GMa.

CK-1 sequences respond to TNF-α but not PMA in fibroblasts. We have shown here that the CK-1 sequence from the G-CSF gene is a TNF-α-responsive enhancer in embryonic fibroblasts. The NF-κB site from both the IL-2R α chain gene, the HIV enhancer, or the immunoglobulin κ gene are not only TNF-α responsive (25, 38) but also respond to phorbol ester in T cells and fibroblasts (38, 42). It appears, however, that the CK-1 sequence is not required for phorbol ester induction of the GM-CSF promoter in T cells (32). We have tested multiple copies of the CK-1 sequence from G-CSF cloned in either orientation upstream of the tk promoter (Fig. 6A) for their ability to respond to either TNF-α or PMA following transfection into primary embryonic fibroblasts. As shown above, multiple copies of the CK-1 sequence can respond to TNF-α and they are also functional in either orientation upstream of the tk promoter in pBLCAT2 (Fig. 6B). However, PMA treatment of cells containing these constructs showed no CAT activity after either 6 or 16 h of exposure to PMA (Fig. 6B). A plasmid containing four copies of the IL-2 NF-κB site (Fig. 4A) showed a weak response to PMA in these cells (data not shown). The CK-1 sequence was also unresponsive to PMA in the 5637 bladder carcinoma cell line, in which we have previously shown that NF-GMa-binding activity does not change with PMA treatment (43) (Fig. 6B). The same concentration (20 ng/ml) of PMA can, however, induce both NF-GMb-binding activity (43) and GM-CSF mRNA in 5637 cells (Fig. 6C) and also both GM-CSF and G-CSF in embryonic fibroblasts (22).

DISCUSSION

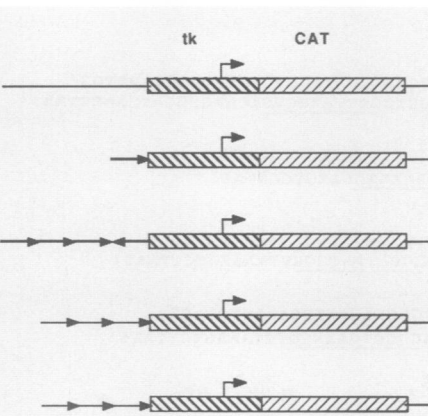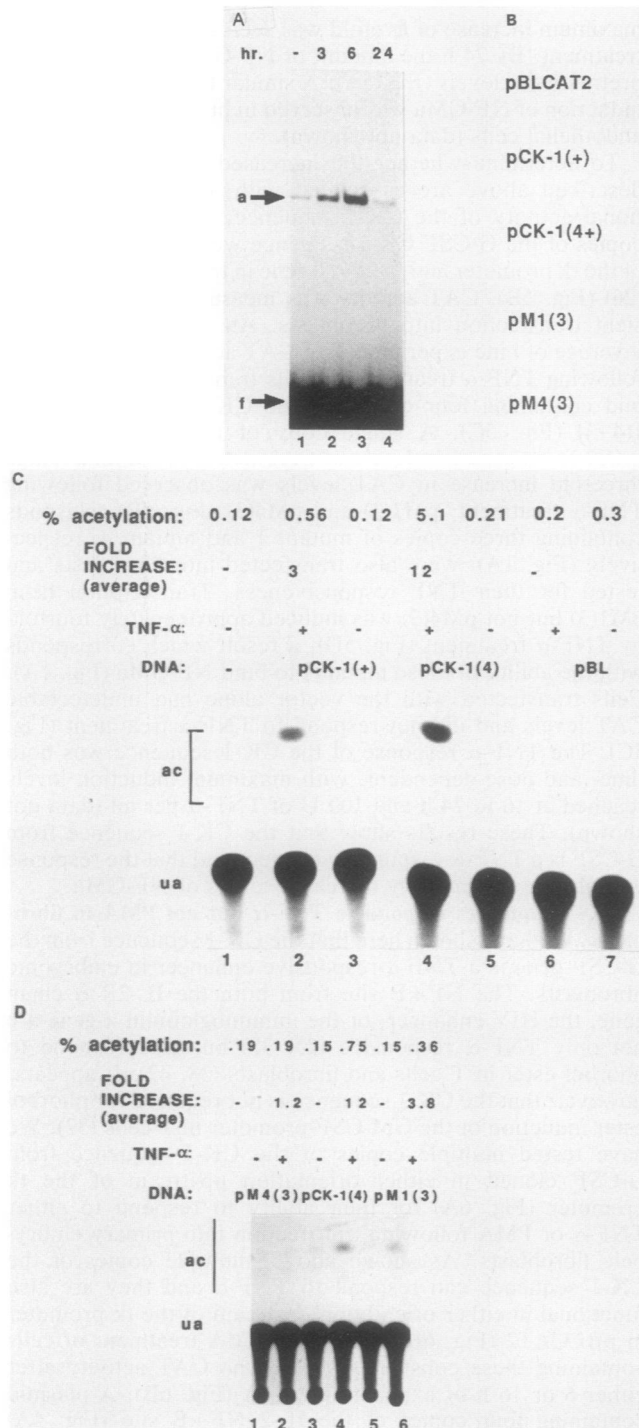The transcriptional regulation of HGF gene expression is likely to be complex, differing from one cell type to another

FIG. 5. (A) NF-GMa induction in fibroblasts by TNF-α. Nuclear extracts were prepared from embryonic fibroblasts following different times of TNF-α treatment. Retardation assays were performed by using the radiolabeled G-CSF CK-1 probe with equal amounts of protein from untreated cells (lane 1) and cells treated with TNF-α (100 U/ml) for 3 h (lane 2), 6 h (lane 3), and 24 h (lane 4). (B) Schematic representation of plasmids containing the G-CSF CK-1 oligonucleotides cloned upstream of the tk promoter (−105 to +51) linked to the CAT gene in pBLCAT2 (26). pCK-1(+) and pCK-1(4) represent one and four copies of the oligonucleotides, respectively. pM1(3) and pM4(3) are plasmids with three copies of mutant 1 and mutant 4, respectively (see Fig. 2). The arrows show the number and direction of cloning of the oligonucleotides. (C) Fibroblasts were mock transfected (lane 1) or transfected with pCK-1(+) (lanes 2 and 3), pCK-1(4) (lanes 4 and 5) and pBLCAT2 (lanes 6 and 7). Twenty-four hours following transfection, cells were left untreated (−) or were treated (+) with TNF-α (100 U/ml) for 16 h prior to harvesting. Cytoplasmic extract (50 μg) was assayed for 4 h for CAT activity. The average "fold" induction by TNF-α for at least four experiments and the percent CAT acetylation for this experiment are shown for each plasmid. Ac indicates acetylated chloramphenicol and ua is the unconverted form. (D) Fibroblasts were transfected as described above with pM4(3) (lanes 1 and 2), pCK-1(4) (lanes 3 and 4), and pM1(3) (lanes 5 and 6). CAT assays were performed (25 μg of cytoplasmic extract for 2 h) on extracts from untreated (−) or TNF-α-treated (+) cells as described in panel C. The average fold induction by TNF-α for four experiments and the percent CAT conversion for this experiment are shown.

and involving the interaction of a number of regulatory nuclear proteins. We have characterized one of these nuclear factors with a binding specificity apparently restricted to HGF genes and which appears to mediate TNF-α inducibility in fibroblasts. This protein, NF-GMa, appears as a 43-kDa protein from several cell types. Mutation analyses and modification interference data both show that the CK-1 sequence is the binding site for NF-GMa. NF-GMb clearly represents a distinct protein which binds to the adjacent CK-2 sequence.

These two proteins can bind independently to DNA when separated by heparin-Sepharose chromatography (43). We have not however detected complexes on retardation gels which contain both proteins, implying that their binding may be mutually exclusive. This possibility is supported by the modification interference data where the main contact points for the two proteins are on the same strand of the DNA, within six bases of each other, and so some interference in binding may occur.

Fragments of DNA spanning the CK-1 sequence from four different HGF genes each bound NF-GMa but with different affinities. Sequences both within and flanking the CK-1 sequence may play some role in this altered affinity. The G-CSF and GM-CSF sequences have identical CK-1 sequences but quite divergent flanking sequences and an approximately twofold difference in affinity for NF-GMa. The IL-3 CK-1 sequence appears to have the highest affinity, and IL-5 has the lowest affinity for NF-GMa. The IL-5 sequence is one of the least conserved CK-1 regions, with three base changes, when compared with GM-CSF CK-1 (Table 1). A series of 3-bp substitutions within the 3′ flanking sequence of the GM-CSF CK-1 region (i.e., across the CK-2 region) did not significantly alter NF-GMa binding (44).
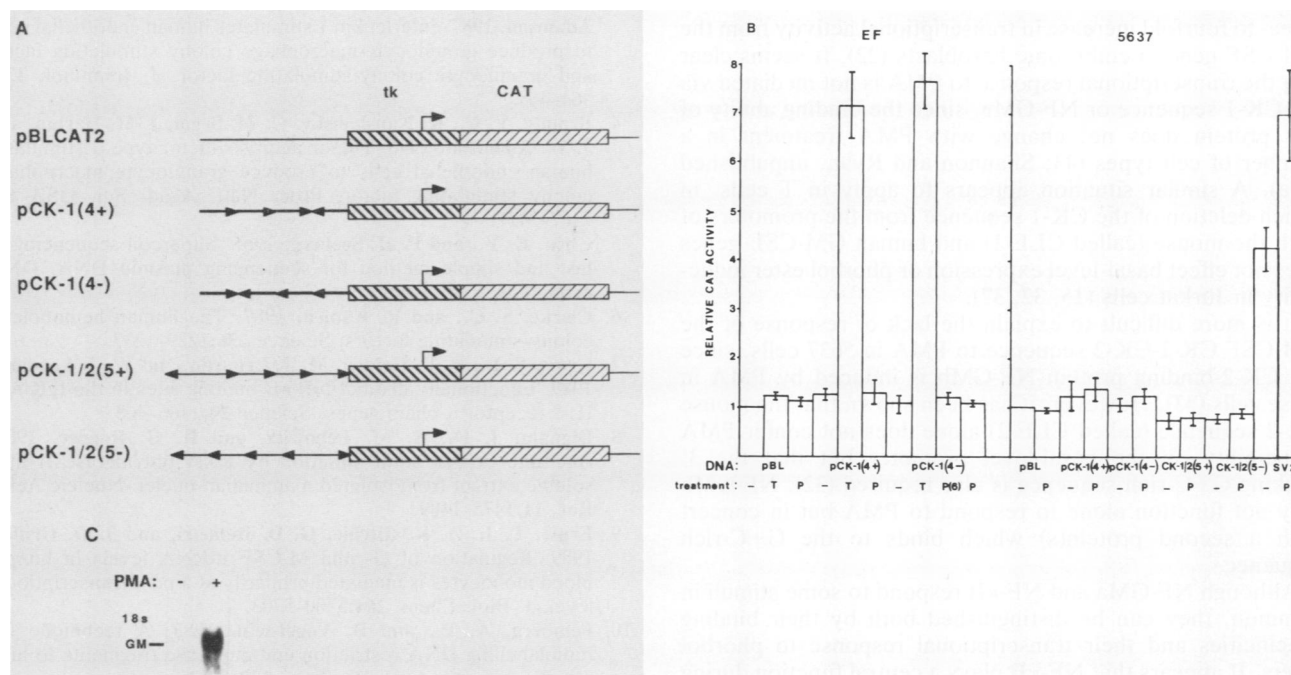
FIG. 6. The CK-1 sequence is TNF-α but not PMA responsive. (A) Schematic representation of plasmid constructs containing multiple copies of either CK-1 from G-CSF [pCK-1(4)] or CK-1/2 from GM-CSF [pCK-1/2(5)] cloned in either orientation (+ and −) upstream of the tk promoter (−105 to +51) linked to the CAT gene in pBLCAT2. The arrows show the number of copies and the direction of the sequences. (B) CAT activity measured in either embryonic fibroblasts of 5637 cells following transfection with the plasmids in panel A. Cells were treated 24 h after transfection with either TNF-α (T) (100 μ/ml) or PMA (P) (20 ng/ml) for 12 to 16 h prior to harvesting. CAT activity was measured and is expressed relative to the level of CAT activity in unstimulated cells transfected with the pBLCAT2 plasmid. The figures shown are the mean (± standard error) of at least three separate transfection experiments. Absolute CAT conversion was on average 4.5% for pCK-1(4+) following TNF-α treatment. (C) Northern blot analysis of mRNA prepared from 5637 cells either untreated (−) or treated with PMA (20 ng/ml) for 6 h (+). Total cytoplasmic RNA (15 μg) was loaded per track. Equivalent tracks were stained with ethidium bromide to ensure equal amounts of RNA in each sample. The ≈1-kilobase GM-CSF mRNA and the position of the 18s ribosomal RNA band are indicated. The blot was probed with a 650-bp GM-CSF cDNA fragment labeled to approximately 1×10^9 cpm/μg by random priming.

Single-base changes within the NF-GMa transcription factor-binding site or perhaps altered flanking sequences can, therefore, significantly alter nuclear factor-binding affinity and ultimately transcriptional efficiency of the DNA fragment.

The physiological significance of the conservation of this sequence across the family of HGF genes and the changes in affinity between different genes for NF-GMa binding is not clear but suggests a mechanism for coregulation. There is no evidence for the coordinate regulation of all the CK-1-containing HGF genes. There is, however, some evidence for the coordinate induction of subsets of these genes. For example, GM-CSF, G-CSF, and IL-6 mRNAs are induced following TNF-α treatment of fibroblasts and endothelial cells (40, 53) and some mouse T-cell clones coordinately express GM-CSF and IL-3 (20). We have shown that the CK-1 sequence from the G-CSF gene is a TNF-α-responsive element in fibroblasts. Levels of NF-GMa-binding activity are also induced by TNF-α and probably function in mediating the response of the CK-1 sequence to TNF-α. This sequence may be involved, therefore, in regulating the expression of several CK-1-containing genes in cells exposed to monocyte products such as TNF-α. However, all CK-1-containing genes are not TNF-α responsive and IL-3 mRNA cannot be detected in endothelial cells following TNF-α treatment (35). In addition, IL-1 induction of GM-CSF in endothelial cells is not affected by deletion of the CK-1 sequence (18), although basal-level expression is altered. The promoter context in which the CK-1 sequence

occurs may be important in controlling its function, and adjacent binding proteins may block CK-1/NF-GMa activity. Such a situation has been described for the NF-κB binding sites from the immunoglobulin κ gene and the IL-2R α-chain gene (7). Although these sequences appear to bind NF-κB with equal affinity, the IL-2R sequence does not respond to phorbol esters in Jurkat T cells and so in vitro binding cannot be equated with in vivo activity (7).

We compared the binding specificity of NF-GMa with that of another TNF-α-responsive transcription factor, NF-κB (17, 25, 38). In spite of the sequence similarity between the NF-GMa- and NF-κB-binding sites, especially those present in the GM-CSF (NF-GMa) and IL-2 (NF-κB) genes (Table 1), our experiments showed that each transcription factor has a distinct set of binding sites and that NF-GMa binding appears to be confined to HGF-type genes. DNA binding and transcriptional activity of NF-κB is induced by PMA in both fibroblasts (41) and T cells (34, 36). Costimulation of Jurkat cells with TNF-α and PMA leads to superinduction of NF-κB (36), implying that these two agents act through independent mechanisms.

Unlike NF-κB-binding sites, the CK-1 sequence from G-CSF did not respond to phorbol ester treatment in embryonic fibroblasts. Similar results were obtained in 5637 cells, in which the CK-1 sequence from both G- and GM-CSF was not PMA inducible. In both of these cell types, G- and GM-CSF mRNA levels are increased following PMA treatment (Fig. 5C [22]). Part of this response is at the level of transcription, since it has been reported that PMA induces a

three- to fourfold increase in transcriptional activity from the GM-CSF gene in embryonic fibroblasts (22). It seems clear that the transcriptional response to PMA is not mediated via the CK-1 sequence or NF-GMa, since the binding ability of this protein does not change with PMA treatment in a number of cell types (43; Shannon and Ryan, unpublished data). A similar situation appears to apply in T cells, in which deletion of the CK-1 sequence from the promoters of both the mouse (called CLE-1) and human GM-CSF genes does not effect basal-level expression or phorbol ester inducibility in Jurkat cells (15, 32, 37).

It is more difficult to explain the lack of response of the GM-CSF CK-1-CK-2 sequence to PMA in 5637 cells, since the CK-2-binding protein NF-GMb is induced by PMA in these cells (43). However, it has been shown that the mouse CK-2 sequence (called CLE-2) alone does not confer PMA inducibility on the basal-level promoter but that the 3' flanking G+C-rich sequence is also required (32). NF-GMb may not function alone to respond to PMA but in concert with a second protein(s) which binds to the G+C-rich sequence.

Although NF-GMa and NF-κB respond to some stimuli in common, they can be distinguished both by their binding specificities and their transcriptional response to phorbol esters. It appears that NF-κB plays a central function during T-cell activation in the induction of IL-2 and the IL-2R α-chain genes (16, 25), although the role played by the κB site alone in the IL-2R sequence is unclear (7). On the other hand, the NF-GMa-binding site does not have a clear role in T cells (32, 37) but could play an important role in induction of G-CSF, GM-CSF, and other HGF genes in fibroblasts. The TNF-α response appears to be cell specific also, since the CK-1 sequence does not respond to TNF in Jurkat T cells (Shannon et al., unpublished data). This function may be relevant both in the bone marrow stroma and at sites of inflammation in local inflammatory responses. In the bone marrow stroma, fibroblasts or endothelial cells may respond to extracellular stimuli via NF-GMa to produce HGF proteins required for steady-state hemopoiesis. TNF produced by infiltrating monocytes could augment the inflammatory response by inducing localized cells to produce HGF proteins, which in turn could activate the cytotoxic or phagocytic functions of peripheral granulocytes or macrophages (24, 29, 48). Cloning of the gene for NF-GMa, an apparently HGF-specific transcription factor, will enable us to determine in more detail its relationship with NF-κB and to elucidate signal transduction mechanisms involved in its activation by TNF-α or other agents.

ACKNOWLEDGMENTS

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