A sequence-specific single-strand DNA binding protein that contacts repressor sequences in the human GM-CSF promoter

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ABSTRACT

NF-GMb is a nuclear factor that binds to the proximal promoter of the human granulocyte – macrophage colony stimulating factor (GM-CSF) gene. NF-GMb has a subunit molecular weight of 22 kDa, is constitutively expressed in embryonic fibroblasts and binds to sequences within the adjacent CK-1 and CK-2 elements (CK-1/CK-2 region), located at approximately – 100 in the GM-CSF gene promoter. These elements are conserved in haemopoietic growth factor (HGF) genes. NF-GMb binding requires the presence of repeated 5'CAGG3' sequences that overlap the binding sites for positive activators. Surprisingly, NF-GMb was found to bind solely to single-strand DNA, namely the non-coding strand of the GM-CSF CK-1/CK-2 region. NF-GMb may belong to a family of single-strand DNA binding (ssdb) proteins that have 5'CAGG3' sequences within their binding sites. Functional analysis of the proximal GM-CSF promoter revealed that sequences in the – 114 to – 79 region of the promoter containing the NF-GMb binding sites had no intrinsic activity in fibroblasts but could, however, repress tumour necrosis factor-α (TNF-α) inducible expression directed by downstream promoter sequences (– 65 to – 31). Subsequent mutation analysis showed that sequences involved in repression correlated with those required for NF-GMb binding.

INTRODUCTION

Granulocyte – macrophage colony stimulating factor (GM-CSF) is one of a family of haemopoietic growth factors (HGF) which control the survival, proliferation and differentiation of haemopoietic progenitor cells as well as the functional activation of mature cells (1 – 5). GM-CSF is produced by many cell types including, mesenchymal cells (e.g. fibroblast and endothelial cells) in response to proinflammatory agents such as tumour necrosis factor (TNF) and interleukin-1 (IL-1) (6 – 8), monocytes in response to endotoxin (9,10) and activated T cells (11,12). Transcriptional regulation plays a major role in both stimulus

and cell-specific expression of GM-CSF (1,13,14). Analysis of the mouse GM-CSF promoter in T cells in response to PMA/ionophore and human T-cell leukaemia virus type I (HTLV-I) tax stimulation has led to the identification of several promoter elements required for function (15 – 18). These include the linked CK-1 and CK-2 elements (the CK-1/CK-2 region), a GC-rich element (GC box) and repeated 5'CATTA/T3' sequences. The 5'CATTA/T3' elements also have a functional role in the human GM-CSF promoter in both T and endothelial cells (14,19 – 22). We have found that the human GM-CSF CK-1/CK-2 region and a binding site for the NF-κB transcription factor, which overlaps the GC-rich element, are required for T-cell response to HTLV-I tax (23). A role for the CK-1/CK-2 region, the NF-κB site and the GC-rich element in mesenchymal cells has not yet been reported.

We have previously reported that the CK-1/CK-2 region (– 114 to – 79) in the human GM-CSF gene binds two nuclear factors, NF-GMa and NF-GMb (24 – 26). NF-GMa bound to sequences spanning the CK-1 elements from a number of HGF gene promoters while NF-GMb bound only to the GM-CSF CK-1/CK-2 region. NF-GMa has been purified and its binding to the CK-1 element of the granulocyte – colony stimulating factor (G-CSF) gene, correlated with the ability of a promoter fragment containing the CK-1 element to respond to TNF-α and IL-1β in human embryonic lung (HEL) fibroblasts (25,27). In contrast the GM-CSF CK-1/CK-2 region does not respond to TNF-α or IL-1β in HEL fibroblasts (27). In addition the TNF-α inducible NF-κB-p65 transcription factor can bind to the CK-1 element of both the G-CSF and GM-CSF genes (23,28) but transactivates only the G-CSF sequence (28). It was considered that the binding of transcription factors to the CK-1/CK-2 region in the GM-CSF promoter could be modulated by the binding of the unique NF-GMb factor. NF-GMb was further investigated to determine its role in GM-CSF promoter regulation.

In this paper we present evidence that full NF-GMb binding requires the presence of repeated 5'CAGG3' sequences in the CK-1/CK-2 region of the GM-CSF promoter and that NF-GMb binds only to single-strand DNA. Evidence is provided that NF-GMb is involved in repression of the CK-1/CK-2 region. The

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CK-1/CK-2 region was also found to repress the TNF-α response of downstream promoter sequences (−65 to −31) and the ability to bind NF-GMb was correlated with this repression effect.

MATERIALS AND METHODS

Cell culture

Human embryonic lung fibrobasts (HEL; Commonwealth Serum Laboratories, Australia) were grown in DMEM and 10% fetal calf serum (FCS). These cells were used from passage 14 to 20 in all experiments. Cells were treated with TNF-α (100 U/ml) for 24 h in transfections and for 6 h for nuclear extractions.

Preparation of nuclear extracts, protein gel slice analysis and UV crosslinking

HEL fibroblasts were grown to 70−80% confluence and nuclei prepared according to the method of Dignam *et al.* (29). Nuclear proteins were prepared as previously described (24). Heparin-sepharose (HS) enriched NF-GMb from the HUT78 T-lymphoblastoid cell line, and crude HUT78 nuclear extracts were prepared as previously described (26).

To size proteins by gel slice analysis, HS fractions of HUT78 nuclear extracts, enriched for NF-GMb, were run on 12.5% SDS−polyacrylamide gels according to the Laemmli method (30), under non-reducing conditions. Successive slices were cut from the gel and the protein eluted and renatured according to the method of Baeuerle and Baltimore (31). Specific DNA binding was monitored using gel retardation analysis.

For UV crosslinking nuclear extracts were bound to single-strand oligonucleotides in a 50 ml retardation reaction and fractionated on a non-denaturing 12% 0.5×TBE polyacrylamide gel (as described below). The gel was exposed to UV light (340 nm) for 15 min and retarded complexes excised after exposure of the gel to X-ray film. Protein in excised bands was analysed on 12% SDS−polyacrylamide gels under reducing or non-reducing conditions as described (25).

Oligonucleotide probe preparation and gel retardation analysis

All oligonucleotides were synthesized on an Applied Biosystems model 381A DNA synthesizer. Full length oligonucleotides (Figs 1and 5) were purified from non-denaturing polyacrylamide gels (32). For gel retardation assays using double-strand (ds) probes, complementary oligonucleotides were end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase (PNK), and then annealed and gel purified. Single-strand (ss) DNA probes were prepared by end-labelling coding (+) or non-coding (−) strand oligonucleotides followed by gel purification.

Gel retardation assays were performed using 0.25 ng of ss or 0.5 ng of ds probes in a 10 μl reaction mix of 0.5×TM (24) buffer containing 200 mM KCl, 0.4 μg polydI:dC and either 0.2 μg of HS HUT 78 material enriched for NF-GMb or 1.0 μg of crude HEL fibroblast or crude HUT78 nuclear extracts. Reactions were incubated at room temperature for 20 min and analysed on 12% non-denaturing polyacrylamide gels in 0.5×TBE (24).

Plasmid constructs

Complementary oligonucleotides for cloning were phosphorylated with T4 PNK and annealed to yield ds DNA. Oligonucleotides with *Eco*RI ends (GM, GMm23, G, G/GM, GM/G, GM-33 and

Figure 1. Oligonucleotides used for gel retardation. Sequence of the coding (+) and non-coding (−) GM oligonucleotide strands (−114 to −79) are shown. The sequences of mutant GM oligonucleotides, G and hybrid G/GM and GM/G oligonucleotides are also shown (coding strands). For mutant GM oligonucleotides only those bases that differ from the wild-type GM sequence are given. The constructs derived from cloning oligonucleotides into pBLCAT2 are listed. The number of oligonucleotide inserts in each construct is given in brackets.

GM-41; Figs 1 and 5) were first cloned into Bluescript SK(−). Inserts from clones containing either single or multiple copies of oligonucleotide sequences were excised with *Bam*HI and *Hin*dIII and cloned upstream of the herpes simplex virus (HSV) thymidine kinase (tk) promoter of the pBLCAT2 reporter plasmid (33; Figs 1 and 5). Oligonucleotides with both *Hin*dIII (5′) and *Bam*HI (3′) ends (GM-43, GM-45, GM-43m1, GM-43m2, GM-43m3, GM-43m4 and GM-43m5) were cloned directly into pBLCAT2 (Fig. 5).

Transfections

HEL fibroblasts were transfected with 10 μg of reporter constructs using DEAE-dextran as described (27). Twenty-four hours following transfection, cells were stimulated with TNF-α (100 U/ml) or left untreated, for an additional 24 h. Cells were then harvested and CAT (chloramphenicol acetyltransferase) assays performed as described (27). Percentage [^{14}C]chloramphenicol conversion to acetylated forms via CAT activity in extracts was determined using phosphorimager analysis (Molecular Dynamics). Relative CAT activity was determined within each experiment using a value of 1.0 for the unstimulated pBLCAT2 vector.

RESULTS

NF-GMb is a sequence-specific single-strand DNA binding protein

We have partially purified NF-GMb binding activity from HUT78 T cells by heparin-sepharose (HS) chromatography (24). We showed by gel retardation using double-strand (ds) oligonucleotides containing the human GM-CSF CK-1/CK-2 region (GM; Fig.1) that mutations in a 5′CAGG3′ sequence within the CK-2 element reduced NF-GMb binding (26). Surprisingly, we also found that NF-GMb bound single-strand DNA. The binding was to the non-coding (−) strand of the GM oligonucleotides, as determined by binding to single-strand (ss) oligonucleotides (Fig. 2a). No binding was observed to the coding (+) strand. Analysis of binding to mutant oligonucleotides showed that full NF-GMb binding required the presence of not
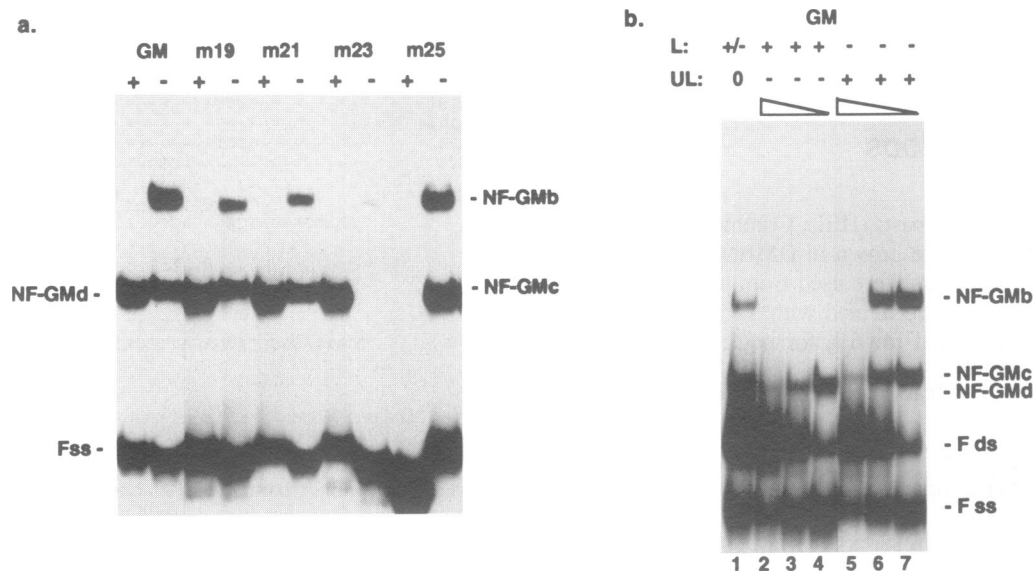
Figure 2. NF-GMb binds only to single-strand DNA. (a) NF-GMb enriched from HUT78 T cells was assayed by gel retardation with GM and mutant GM single-strand (ss), coding (+) and non-coding (−), oligonucleotides (Fig. 1). (b) Gel retardation was performed, as for (a), using ds probe mixes with only one strand, either the coding (+) (tracks 2−4) or non-coding (−) (tracks 5−7), of the GM oligonucleotide labelled (L). In track 1 both strands are labelled. The amount of unlabelled (UL) strand in probe mixes decreases from tracks 2−4 and from tracks 5−7 by a factor of 4. Complexes and free ds (Fds) and single-strand (Fss) probes are indicated.

only the CK-2 5'CAGG3' sequence but also a 5'CAGG3' at the 5' end of the CK-1 element. Mutation of either of the 5'CAGG3' elements reduced NF-GMb binding (m19, m21) while mutation of both 5'CAGG3' (m23) essentially abolished NF-GMb binding. Mutation of repeated 5'TTC3' sequences (m25) flanking the 5'CAGG3' sequences did not affect NF-GMb binding (Fig. 2a).

As ds probes used to detect NF-GMb binding contain a percentage of ss DNA, we wished to determine if NF-GMb bound exclusively to ss DNA or if it bound both ds and ss DNA. To do this ds probes were made with only one strand, either the coding (+) or non-coding (−) strand, of the GM oligonucleotides labelled. If NF-GMb bound ds DNA as well as ss DNA, complex formation would be apparent regardless of which strand was labelled. If the protein bound solely to ss DNA, a complex would only be apparent in probe mixes containing the appropriate labelled ss DNA. This is what was observed, with NF-GMb complex formation being apparent only when probes contained labelled non-coding (−) strand DNA (Fig. 2b). Consistent with this, decreasing the amount of unlabelled coding (+) strand in probe mixes and thereby increasing the relative amounts of the free labelled non-coding (−) strand, resulted in increasing the amount of NF-GMb complex formed (Fig. 2b, tracks 5−7).

Complexes formed by the binding of two additional single-strand DNA binding (ssdb) factors, NF-GMc and NF-GMd, were also apparent in gel retardation assays (Fig. 2a). NF-GMc also bound to the non-coding (−) strand of the GM sequence and its binding was abolished when both 5'CAGG3' sequences were mutated (Fig. 2a). NF-GMc binding, however, was not affected by mutating either 5'CAGG3' sequence alone. The NF-GMc complex appears to be the result of protein binding to either the CK-1 or CK-2 5'CAGG3' sequence but not both simultaneously. NF-GMd bound exclusively to the coding (+) strand, and its binding can be localized to the 5'TTCC3' within the CK-1

element (data not shown). NF-GMc and NF-GMd also bound only to single-strand DNA (Fig. 2b). NF-GMc and NF-GMd unlike NF-GMb also bind to the G-CSF CK-1 region (data not shown).

NF-GMb has a subunit size of 22 kDa

To determine the molecular weight of NF-GMb, HS-enriched material from HUT78 T cells was fractionated on an SDS−polyacrylamide gel and broad slices taken down the length of the gel. Eluted, renatured protein from slices was analysed by gel retardation. NF-GMb binding activity was identified in a protein fraction of approximately 20 kDa (data not shown). A finer gel slice analysis was performed in the size range 14−30 kDa. This separated NF-GMb from other binding activities and showed NF-GMb to be 22 kDa (gel slice 7, Fig. 3a). The other binding proteins (X, X*) are as yet uncharacterized.

NF-GMb-enriched HUT78 material bound to the non-coding (−) GM oligonucleotide was also subjected to UV crosslinking. Crosslinked NF-GMb and NF-GMc complexes were fractionated on reducing and non-reducing SDS−polyacrylamide gels (Fig. 3b). The size of proteins in crosslinked complexes was determined by subtracting the apparent size of the free DNA probe from the size of the DNA−protein complex. In agreement with the gel slice experiment a major protein band of 22 kDa was observed after crosslinking the NF-GMb complex (band 2). A larger protein band of 42.5 kDa was also observed (band 1). Band 1 was seen consisently on both reducing and non-reducing gels, although amounts of band 1 varied between experiments (compare Fig. 4b). Sizing of the NF-GMc complex revealed a single band of 22 kDa. It is possible that NF-GMc represents the binding of a single 22 kDa subunit to the CK-1/CK-2 region and that the NF-GMb complex represents the binding of two 22 kDa subunits to the CK-1/CK-2 region. This is consistent with
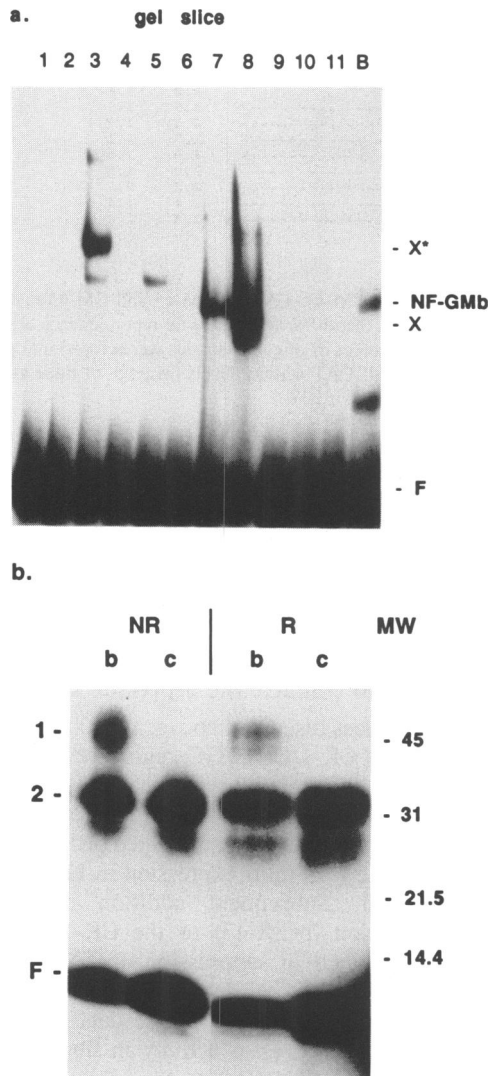
a.   gel slice

b.

a.

b.

Figure 3. NF-GMb has a subunit molecular weight of 22 kDa. (a) Gel retardation assay of gel slice material from 30 to 14 kDa. NF-GMb enriched from HUT78 T cells was fractionated on non-reducing SDS—polyacrylamide gels and protein from gel slices was assayed by gel retardation using ds GM oligonucleotides. Tracks 3, 7 and 8 represent material of 27, 22 and 20 kDa respectively. Track B is unfractionated, enriched NF-GMb from HUT78 T cells. Specific complexes and free probe (F) are indicated. (b) UV crosslinking analysis of NF-GMb. NF-GMb (b) and NF-GMc (c) complexes, from enriched HUT78 material bound to non-coding (−) strand GM oligonucleotides, were irradiated with UV light and analysed on non-reducing (NR) and reducing (R) SDS—polyacrylamide gels. Free probe (F) and proposed dimer (1) and monomer (2) protein bands are indicated. The sizes of molecular weight markers (MW) are in kilodaltons. Protein sizes were determined by subtracting the size of the free probe (13.5 kDa).

Figure 4. NF-GMb expression in fibroblasts. (a) Gel retardation assay using ss GM oligonucleotides was performed with NF-GMb enriched from HUT78 T cells (B), with unstimulated (HEL−T) and TNF-α stimulated (HEL+T) HEL fibroblast crude nuclear extracts and HUT78 crude nuclear extracts (HUT). (b) UV crosslinking was carried out on NF-GMb complexes formed from binding of enriched NF-GMb from HUT78 T cells (B), crude HUT78 nuclear extracts (HUT), and crude TNF-α stimulated HEL fibroblast nuclear extracts (HEL), to non-coding (−) strand GM oligonucleotide as described in Fig. 3. Crosslinked complexes were analysed on reducing SDS—polyacrylamide gels. Proposed dimer (1) and monomer (2) protein bands and free probe (F) are indicated.

retardation results that determined that the NF-GMb complex represented simultaneous protein binding to both 5′CAGG3′ elements and that NF-GMc represented binding to one or other 5′CAGG3′ element. Any linkage between the two subunits in the NF-GMb complex must be readily disturbed on SDS—polyacrylamide gels, as a 42.5 kDa protein is not detected by gel slice analysis and is not the primary complex observed after gel analysis of UV crosslinked material.
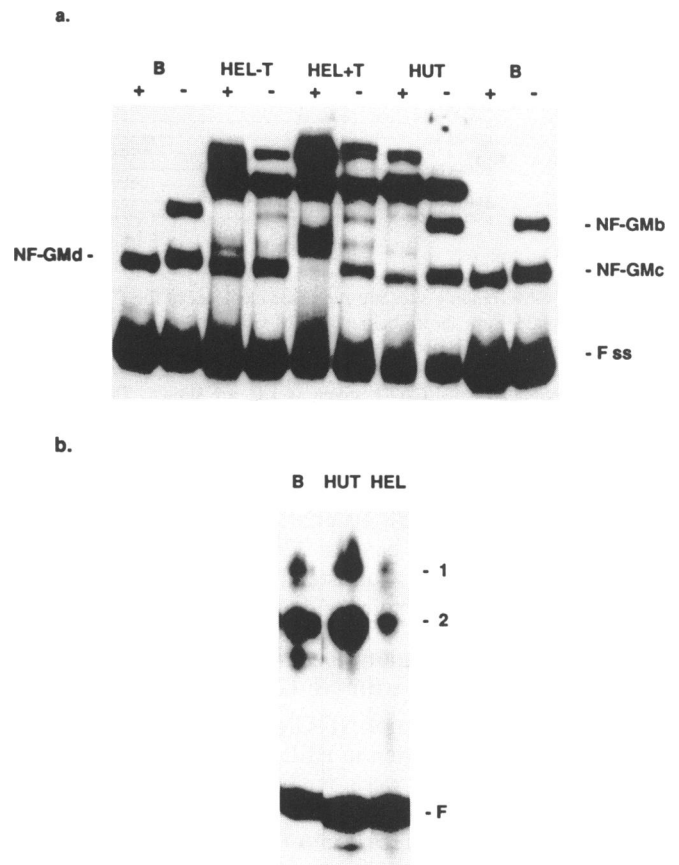
NF-GMb is expressed in HEL fibroblasts

As we wished to determine the role of NF-GMb in GM-CSF promoter regulation in fibroblasts, gel retardations were performed to verify the presence of NF-GMb in these cells. Complexes migrating with the same mobility as the NF-GMb, NF-GMc and NF-GMd complexes, from HS-enriched HUT78 material, were seen in both TNF-α stimulated and unstimulated crude nuclear fibroblast (human embryonic lung, HEL) extracts (Fig. 4a). Unfractionated crude HUT78 nuclear extracts were also assayed for comparison. Fibroblast NF-GMb bound in the same manner to mutant GM oligonucleotides as HUT78 NF-GMb (data not shown). The amount of NF-GMb binding activity is less in fibroblasts than in HUT78 extracts but the significance of this at present is unclear. GM-CSF promoter activity will have to be compared in the two cell lines. To further confirm the presence of NF-GMb in HEL fibroblasts UV crosslinking was performed (Fig. 4b). As for NF-GMb complexes from heparin-
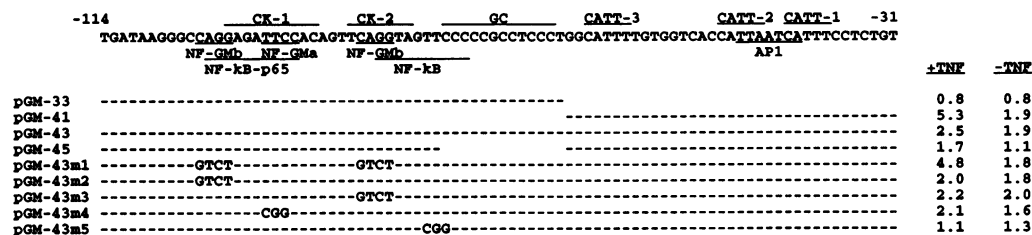
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        -114              CK-1        CK-2         GC         CATT-3        CATT-2 CATT-1   -31
      TGATAAGGGCCAGGAGATTCCACAGTTCAGGTAGTTCCCCCGCCTCCCTGGCATTTTGTGGTCACCATTAATCATTTCCTCTGT
           NF-GMb   NF-GMa    NF-GMb                                              AP1
             NF-kB-p65               NF-kB                                                        +TNF    -TNF

pGM-33   ------------------------------------------------                                          0.8    0.8
pGM-41                                                          -----------------------------     5.3    1.9
pGM-43   ------------------------------------------------      -----------------------------     2.5    1.9
pGM-45   -------------------------------------                 -----------------------------     1.7    1.1
pGM-43m1 -----------GTCT-------------GTCT----------------      -----------------------------     4.8    1.8
pGM-43m2 -----------GTCT-------------------------------        -----------------------------     2.0    1.8
pGM-43m3 --------------------------GTCT------------------      -----------------------------     2.2    2.0
pGM-43m4 -----------------CGG-----------------------------     -----------------------------     2.1    1.6
pGM-43m5 -----------------------------CGG-----------------     -----------------------------     1.1    1.3
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Figure 5. NF-GMb binding sites are repressor elements of the CATTA/T region. The sequences of GM-33, GM-41, GM-43, GM-45 and GM-43 mutant (m1 to m5) oligonucleotides are shown below the GM-CSF promoter sequence. Only those bases of oligonucleotides that differ from the wild-type sequence are indicated. The sequence in GM-45 is not contiguous and represents bases −114 to −79 joined to −65 to −31. Single copies of oligonucleotides were cloned into pBLCAT2, transfected into HEL fibroblasts, followed by treatment with or without TNF-α and CAT activity determined. CAT activity levels (average of three experiments) relative to unstimulated pBLCAT2, given as 1.0, are shown. The given values have standard errors of less than 20%.

sepharose enriched HUT78 material and HUT78 crude nuclear extracts, a major protein band of 22 kDa and a minor band of 42.5 kDa were detected. Consistently, the fibroblast NF-GMc complex gave a single protein band of 22 kDa (data not shown).

The CK-1/CK-2/GC region does not respond to TNF-α in fibroblasts—correlation with NF-GMb binding

To determine the function of NF-GMb in the regulation of the GM-CSF CK-1/CK-2 region in HEL fibroblasts, wild-type and mutant oligonucleotides were multimerized and linked to a heterologous promoter on the CAT reporter gene (pBLCAT2 vector, Fig. 1) and transfected into HEL fibroblasts (Table 1). As previously observed (27), the cloned multimerized GM oligonucleotides (pGM; pCK-1/2(5+)) did not respond to TNF-α in fibroblasts, whereas the cloned G oligonucleotides (pG; pCK-1(4+)), containing an identical CK-1 element, did respond to TNF-α. To test if NF-GMb binding accounts for the lack of activity of the GM-CSF CK-1/CK-2 region, the GMm23 oligonucleotides containing mutations in both 5'CAGG3' sites were investigated. The mutation, as shown above, results in loss of NF-GMb binding but did not restore TNF-α responsiveness (pGMm23) to the GM-CSF CK-1/CK-2 region. The GMm23 mutation, however, also results in the loss of binding of the activator NF-κB-p65 whose binding site overlaps the distal 5'CAGG3' element (23). A different approach was then used whereby the GM-CSF CK-2 element was placed adjacent to the G-CSF CK-1 element (G/GM). This resulted in restoring NF-GMb binding and loss of TNF-α inducibility (pG/GM; Table 1). In the inverse experiment replacing sequences 3' of the GM-CSF CK-1 element with sequences 3' of the G-CSF CK-1 element resulted in loss of NF-GMb binding (GM/G) and restored TNF-α inducibility (pGM/G; Table 1). These results therefore provide a correlation between the lack of NF-GMb binding to promoter fragments containing CK-1 elements and TNF-α responsiveness. There is, however, no correlation between CK-1 element response and NF-GMc or NF-GMd binding.

In addition to the NF-κB-p65 site overlapping the 5' NF-GMb site there is an NF-κB(p65/p50) binding site that spans the 3' NF-GMb binding site and the downstream GC-rich element (28,34). The complete NF-κB element was not contained within the GM oligonucleotides used above. An oligonucleotide containing the CK-1/CK-2 and 3' GC-rich elements (GM-33; −114 to −66) did not, however, respond to TNF-α induction

when cloned as a single copy (pGM-33; Fig. 5) or when multerized (data not shown) in pBLCAT2. The CK-1/CK-2/GC region of the GM-CSF promoter by itself, therefore, does not respond to TNF-α in fibroblasts.

The GM-CSF CK-1/CK-2 region represses downstream sequences: NF-GMb contacts the repressor sequences

In contrast to sequences discussed above, functional analysis of sequences 3' of the CK-1/CK-2/GC region revealed that the region −65 to −31 containing three repeated 5' CATTA/T 3' elements (CATTA/T region, GM-41; Fig. 5) could direct both constitutive (1.9-fold relative to unstimulated pBLCAT2 vector) and TNF-α inducible (5.3-fold) expression in HEL fibroblasts (pGM-41; Fig. 5). Subsequent addition of either the CK-1/CK-2/GC region (pGM-43) or the CK-1/CK-2 region (pGM-45) alone resulted in suppression of TNF-α inducible expression directed by the CATTA/T region (reduced to 2.5- and 1.7-fold respectively; Fig. 5). These data imply that the CK-1/CK-2 region has repressor activity in fibroblasts.

To determine the role of NF-GMb binding sites in the repressor activity of the CK-1/CK-2 region, mutations were made within the GM-43 oligonucleotide and mutant sequences cloned into pBLCAT2 (Fig. 5). Mutation of either the CK-1 or CK-2 5'CAGG3' elements alone (pGM-43m2, pGM-43m3), which reduces NF-GMb binding activity *in vitro*, had no effect on expression relative to the wild-type pGM-43 construct. Mutation of both 5'CAGG3' elements, however, which abolishes NF-GMb binding resulted in increased TNF-α inducible expression (pGM-43m1). Mutation of the CK-1 element 5'TTC3' (pGM-43m4) had no effect on expression while mutation of the 5'TTC3' in the downstream NF-κB site resulted in loss of all expression (pGM-43m5). A similar reduction in activity was observed when the GC-rich element, containing the 3' end of the NF-κB site, was deleted from the −114 to −31 region (pGM-45). Sequences within the NF-κB site are probably functioning in cooperation with the downstream CATTA/T sequences, given that the CK-1/CK-2/GC region alone is non-functional in fibroblasts. These experiments therefore demonstrated that by eliminating NF-GMb binding sites it is possible to eliminate the repressor activity of the CK-1/CK-2 region. In contrast the amount of NF-GMb protein binding *in vivo* to the single 5'CAGG3' mutants must be sufficient to allow full repressor activity.

DISCUSSION

A family of ssdb proteins with 5′CAGG3′ recognition motifs

This work describes the characterization of a unique nuclear factor, NF-GMb, that binds the human GM-CSF proximal promoter. In this study we determined that the NF-GMb protein surprisingly bound to single-strand (ss) DNA, namely, to the non-coding strand (−) of the GM-CSF CK-1/CK-2 region (−114 to −79), and that it cannot bind double-strand (ds) DNA. The binding of NF-GMb was sequence-specific and full binding required the presence of two 5′CAGG3′ sequences, within the CK-1/CK-2 region.

Our data suggested that the NF-GMb complex is formed from the binding of two 22 kDa subunits, each contacting a 5′CAGG3′ element, resulting in a 42.5 kDa protein complex, as determined by UV crosslinking. Any linkage between the two subunits must be readily disturbed by SDS gel electrophoresis as a 42.5 kDa band is not observed after gel slice analysis. This is further supported by the lack of NF-GMb complex formation on the hybrid GM/G sequence (Table 1) where the 3′ half of the GM oligonucleotide, containing the CK-2 5′CAGG3′, is replaced by unrelated G-CSF sequences. If the two subunits were linked then the NF-GMb complex should still be observed. Given this, the observation of NF-GMb complex formation on GM mutants with one 5′CAGG3′ mutated suggests that there are additional sequences other than 5′CAGG3′ (or the repeated 5′TTC3′) that the 22 kDa subunit can contact in the wild-type GM sequence allowing NF-GMb complex formation but at a reduced efficiency. Two additional sequence-specific nuclear factors, NF-GMc and NF-GMd, that bound to ss CK-1/CK-2 region DNA were also identified but these factors were not unique to the GM-CSF CK-1/CK-2 region and also bound the G-CSF CK-1 region. Interestingly, NF-GMc appears to represent the binding of a single 22 kDa subunit to one or other 5′CAGG3′ element and therefore is likely to represent the monomer form of NF-GMb.

There have been several recent reports of nuclear proteins with ssdb activity that can contact promoter sequences (35−51). In many cases the precise binding sites for these proteins have not been defined; however, examination of the sequences to which these proteins bind revealed a number of binding sites that contain 5′CAGG3′ sequences (Table 2). These sequences are all within functional promoter elements involved in either activation or repression and lie adjacent to or overlap the binding sites for other nuclear factors.

There is little known of the actual role the ssdb proteins listed in Table 2 play in the regulation of transcription except in the case of the DBSF protein. This factor stimulates the binding of the human oestrogen receptor to an overlapping oestrogen responsive element (ERE). Mutation of the 5′CAGG3′ overlapping the ERE of the vitellogenin A2 gene abolished DBSF and receptor complex formation (42). In contrast, NF-GMb, implicated in repression, could interfere with the binding of positive factors to the GM-CSF promoter, as discussed below. NF-GMb may belong to a family of ssdb proteins that affect either negatively or positively the interaction of transcription factors with DNA. A role for ssdb proteins in gene regulation is also suggested by studies on other ssdb proteins. First, the cellular ssdb protein H16 that contacts the SV40 early promoter can

Table I. NF-GMb binding correlates with lack of CK-1/CK-2 region response to TNF-α

Construct[a]	TNF	Relative CAT activity[b]	NF-GMb binding[c]
pGM (5)	+	0.8	+
	−	0.9	
pGMm23 (3)	+	0.6	−
	−	0.7	
pG (4)	+	12.0	−
	−	0.8	
pG/GM (3)	+	0.7	+
	−	0.9	
pGM/G (3)	+	58.0	−
	−	1.1	
pBLCAT2	+	0.7	
	−	1.0	

[a]Constructs (Fig. 1) were transfected into HEL fibroblasts and cells stimulated with TNF-α (+) or left unstimulated (−).
[b]Relative CAT activity (average of 3 experiments) was determined using a value of 1.0 for unstimulated pBLCAT2.
[c]Results of binding NF-GMb enriched from HUT78 T cells to single-strand GM, GMm23, G, G/GM and GM/G non-coding strand (−) oligonucleotides in gel retardation assays. NF-GMb binding was not observed to coding (+) strands.

Table II. Conserved 5′CAGG3′ elements in single-strand DNA binding protein recognition motifs

Gene[a]	Sequence[b]		Binding Protein	Binding strand
hGM-CSF	a.	+ G C C A G G A G	NF-GMb	−
	b.	+ T T C A G G T A		
mA	a.	+ A C C A G G C A	M1	−
	b.	+ G G C A G G A G		
xVA2		+ G T C A G G T C	DBSF	−
cSA		− C C C A G G A A	MF3	+/−
mCK		− A G C A G G T G	MF3	−

[a]The mouse adipsin (mA; 47), *Xenopus laevis* vitellogenin A2 (xVA2; 42), chicken skeletal actin (cSA; 45) and mouse creatine kinase (mCK; 45) gene 5′CAGG3′ elements on coding (+) and non-coding (−) strands are shown. Elements are compared to the human (h) GM-CSF CK-2 5′CAGG3′.
[b]Conserved bases are marked with a dot. Repeated sequences are marked a. and b.; a. is the most distal element.

Table III. Conserved sequences in negative regulatory elements of immune function genes

Gene	Sequence[a]
hGM-CSF	−89 T T C A G G T A −82
hIL-2	−110 G A C A G G T A −103
hIL-3	−116 G T C A G A T A −109
hIL-2Rα	−385 C C C A G G T G −378
hIFN-β	−49 T T C A G A G G −56

[a]The human interleukin-2 (hIL-2; 60), interleukin-3 (hIL-3; 58), IL-2 receptor α (hIL-2Rα; 61) and interferon-β (hIFN-β; 62) gene elements are compared to the human (h) GM-CSF CK-2 5′CAGG3′. Sequences are coding strand except for hIFN-β. Conserved bases are marked with a dot.

stimulate transcription by RNA polymerase II (38) and secondly two characterized yeast transcriptional regulators MCM1 and α1 have ssdb activity (39).

As for NF-GMb, the DBSF and M1 factors listed in Table 2 bind exclusively to ss DNA. If these proteins are physiologically significant they must therefore acquire access to promoter regions by recognizing DNA in altered or open conformations. Consistent with this hypersensitive sites for the single-strand nuclease S1 have been demonstrated in the promoter regions of a number of genes *in vitro* (48,49,51−55), and single-strand DNA has been demonstrated in the c-*myc* promoter *in vivo* (50). In several reports these sites correspond to binding sites for ssdb proteins (48−51). Hence, ssdb proteins contacting promoter elements no doubt represent an important new group of regulatory factors.

Repression of the CK-1/CK-2/GC region

Transfection experiments revealed a correlation between the lack of CK-1/CK-2 region response to TNF-α and binding of NF-GMb, suggesting that NF-GMb may repress CK-1/CK-2 region function. There was no correlation between lack of TNF-α response and NF-GMc or NF-GMd binding. If NF-GMc represents the monomer form of NF-GMb then dimer formation would be required for repression. The CK-1/CK-2/GC region (−114 to −66) of the GM-CSF promoter, which contains a full binding site for NF-κB immediately 3' to the CK-1/CK-2 region, was also not responsive to TNF-α in fibroblasts. The function of this NF-κB site may also be repressed by NF-GMb.

We propose, that as NF-GMb binds only to single-strand CK-1/CK-2/GC DNA, this will automatically preclude the binding of activators that require ds DNA for binding and hence prevent their action on the CK-1/CK-2/GC region DNA *in vivo*. Such activators may include the TNF-α inducible NF-GMa, NF-κB or NF-κB-p65 activators that can bind to this region *in vitro*. All these proteins are upregulated in HEL fibroblasts (our unpublished data) and the binding sites for these proteins either overlap or are adjacent to the NF-GMb binding sites (Fig. 5).

Repression of sequences downstream of the CK-1/CK-2/GC region

The region of the human GM-CSF promoter containing the three 5'CATTA/T3' sequences (CATTA/T region, −65 to −31) directed both constitutive and TNF-α inducible expression in fibroblasts (pGM-41). Similar results were also found with IL-1β induction (data not shown). This is consistent with work by Kaushansky (14) who demonstrated using consecutive 5' deletions that the region of the human GM-CSF promoter 3' of −63 could direct both constitutive and IL-1β inducible expression in endothelial cells. The region 3' of −53 of the human GM-CSF promoter also directs constitutive and inducible expression in T cells (19−21). This region has recently been shown to bind the TNF-α inducible AP1 transcription factor and the NFAT factor (56; our unpublished data).

Addition of the CK-1/CK-2/GC (−114 to −66) or the CK-1/CK-2 (−114 to −79) sequences to the CATTA/T region (−65 to −31) of the human GM-CSF promoter (pGM-43 and pGM-45 respectively) resulted in suppression of TNF-α induced activity, identifying the CK-1/CK-2 region as a repressor element in fibroblasts. Mutation of the CK-1/CK-2/GC sequences within −114 to −31 demonstrated that NF-GMb binding sites are involved in this repression of downstream sequences. There has only been one other case where specific binding sites for ssdb

proteins have been correlated with repressor activity. This is for the SRE-BF and CNBP ssdb factors that contact the sterol regulatory element within the HMG-CoA reductase gene (44,46). The mechanism of action of these proteins has not been determined.

NF-GMb, bound to ss CK-1/CK-2/GC DNA, could be interacting with CATTA/T region activating transcription factors, such as AP1, and inhibiting their function. NF-GMb could also affect factor binding by bringing about conformational changes in the promoter.

Repressor regions have been identified in a number of HGF and immune-related genes. Interestingly, in many cases these regions contain NF-GMb-like binding sequences (Table 3). In the human interleukin-3 (IL-3) gene, the element shown in Table 3 represents the CK-2 element. Additional sequences in the human IL-3 gene (−397 to −313 and −271 to −250) have repressor activity and also contain NF-GMb-like binding sites (57,58). A common mechanism may exist to maintain an inactive promoter state for many immune function genes.

Given the binding characteristics of NF-GMb, the proximal GM-CSF promoter (3' of −114) analysed here must be able to take on a preferred open or altered conformation to allow NF-GMb binding as discussed above. We predict then, that there must be additional elements further 5' of −114 of the GM-CSF promoter that can modulate NF-GMb binding to allow TNF-α inducible expression in fibroblasts. Recently, a powerful enhancer has been identified, at −3 kb in the human GM-CSF gene that can activate the promoter in T cells (59). Such an enhancer may also function in fibroblasts. The binding of TNF-α inducible factors to such an enhancer could result in the removal of NF-GMb. This could be brought about by an effect on DNA structure placing the CK-1/CK-2/GC region in a preferred double-strand form and displacing NF-GMb.

The analysis of the structure of the GM-CSF promoter *in vivo* and the cloning of a cDNA for NF-GMb will be required to determine the exact mechanism by which NF-GMb functions.

ACKNOWLEDGEMENTS

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