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Cold shock domain proteins repress transcription from the GM-CSF promoter

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ABSTRACT

The human granulocyte-macrophage colony stimulating factor (GM-CSF) gene promoter binds a sequence-specific single-strand DNA binding protein termed NF-GMb. We previously demonstrated that the NF-GMb binding sites were required for repression of tumor necrosis factor-α (TNF-α) induction of the proximal GM-CSF promoter sequences in fibroblasts. We now describe the isolation of two different cDNA clones that encode cold shock domain (CSD) proteins with NF-GMb binding characteristics. One is identical to the previously reported CSD protein dbpB and the other is a previously unreported variant of the dbpA CSD factor. This is the first report of CSD factors binding to a cytokine gene. Nuclear NF-GMb and expressed CSD proteins have the same binding specificity for the GM-CSF promoter and other CSD binding sites. We present evidence that CSD factors are components of the nuclear NF-GMb complex. We also demonstrate that overexpression of the CSD proteins leads to complete repression of the proximal GM-CSF promoter containing the NF-GMb/CSD binding sites. Surprisingly, we show that CSD overexpression can also directly repress a region of the promoter which apparently lacks NF-GMb/CSD binding sites. NF-GMb/CSD factors may hence be acting by two different mechanisms. We discuss the potential importance of CSD factors in maintaining strict regulation of the GM-CSF gene.

INTRODUCTION

The expression of cytokines in specific cell types and in response to specific stimuli is tightly regulated. Control of gene transcription plays a major role in this regulation (1). Numerous transcription factors have been identified that mediate the activation of cytokine genes, including C/EBP, NF-κB, AP1, NF-AT and ets factors (2–4). As well as responding to activators, it is important that cytokine genes can be maintained in a repressed state in the absence of activation signals and that these genes can be completely inactivated upon withdrawal of activation signals. The mechanisms of cytokine gene repression have not been well characterized. Repressor elements have, however, been identified in a number of cytokine and immune function gene promoters, such as in the interleukin-1β (IL-1β), IL-2, IL-3, IL-8, tumor necrosis factor-α (TNF-α), IL-2 receptor-α, interferon-β (IFN-β), LD78, VCAM-1 and ELAM-1 promoters (5–11). In most cases little is known about the nature or function of the proteins binding to these elements. Cloning and characterization of the IRF-2 repressor of IFN-β- and IFN-inducible genes has however demonstrated that it functions by competition with an activator (12). It has also been demonstrated that the repressor of the ELAM-1 gene (ATF-a0) acts by heterodimerizing with an activator and preventing its function (10).

The gene for the cytokine granulocyte-macrophage colony stimulating factor (GM-CSF), is expressed in a wide variety of cells, including fibroblast and endothelial cells, in response to TNF-α and IL-1 and in activated T cells (3,13–15). The proximal promoter region of the GM-CSF gene can be divided into two functional domains (see Fig. 4). The first, containing repeated 5′-CATTA/T-3′ elements (–65 to –31), has been shown to bind AP1, ets and NF-AT transcription factors and to respond to activation signals in fibroblasts, endothelial cells and Jurkat T cells (3,11,13,14,16–19). The second domain (–114 to –66) contains CK-1 and CK-2 sequence elements, which are conserved in many cytokine genes, and is responsive to the HTLV-1 activator, tax and to CD28 co-stimulation in Jurkat T cells (3,18,20). This domain binds a number of nuclear proteins, including activators from the NF-κB family (3,18,20,21). In contrast to the situation in T cells, we have determined that the –114 to –66 domain of the human GM-CSF promoter has repressor activity in fibroblasts (11). This region of DNA can partially repress the TNF-α-inducible response of the downstream –65 to –31 region. The ability of this domain to act as a repressor element correlates with the binding of a nuclear factor called NF-GMb and mutation of the NF-GMb binding sites (within –114 to –79) results in relief of repression of the downstream –65 to –31 region. We also previously presented evidence that NF-GMb binding could be involved in repressing the NF-κB sites across the –114 to –66 region (11). An NF-κB site overlapping the 3′-end of the NF-GMb binding sites (see Fig. 4)

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was found however to be weakly responsive to TNF-α in fibroblasts (11).

We found that NF-GMb from fibroblast and HUT78 T cell nuclear extracts bound only to single-strand DNA and contacted two repeated 5′-CCTG-3′ sequences on the non-coding strand of the GM-CSF –114 to –79 region (see Fig. 2a). Both repeats were required for full NF-GMb binding. We could not detect any binding of nuclear NF-GMb to double-strand DNA. In addition to NF-GMb, we detected a faster migrating nuclear complex, NF-GMc, which results from protein binding to one or other of the repeated 5′-CCTG-3′ sequences on the non-coding strand of the –114 to –79 region. UV cross-linking demonstrates that the NF-GMb complex is composed of a 42.5 and a 22 kDa protein, while the NF-GMc complex is composed of only a 22 kDa protein (11). We previously interpreted this such that NF-GMb represented the binding of a dimer of 22 kDa, with NF-GMc representing the binding of a single 22 kDa monomer (11). It is also possible that NF-GMb represents the binding of two separate protein complexes, both a 42.5 kDa protein and a dimer of the 22 kDa protein. We determined that NF-GMb/c binding activity was constitutively present in nuclear extracts of T cells and fibroblasts (11, unpublished data).

We now report the isolation of cDNA clones which encode proteins with NF-GMb-like binding activity. The cDNAs code for factors belonging to an unusual group of proteins called cold shock domain (CSD) factors. Sequences for two different CSD factors were cloned, one identical to dbpB and the other a potentially new variant of dbpA. We show that the nuclear NF-GMb complex is composed of CSD factors and that overexpression of CSD factors leads to repression of the GM-CSF promoter.

MATERIALS AND METHODS

Expression library screening and phage dot blots

A total of 5×10^5 phage from a λgt11 cDNA expression library, made from HUT78 T cell RNA (Clontech, CA), were screened as previously described (22) with an NF-GMb binding site probe. The binding conditions used were those described for detecting nuclear NF-GMb complex formation in gel retardation assays (11) except that binding was performed at 4°C. The probe was a single-strand DNA oligonucleotide (2×GM–) containing a double copy of the –114 to –79 region of the non-coding (–) strand of the human GM-CSF promoter, which contains the repeated NF-GMb binding sites. The sequence of the 2×GM– probe is: 5′-aattcAACTACCTGAACTGTGGAATCTCCTGGCCCTTA-TCAgaattcAACTACCTGAACTGTGGAATCTCCTGGCCC-TTATCAg-3′ (upper case letters represent GM-CSF promoter sequences and the NF-GMb binding sites are underlined). The 2×GM– probe was ^{32}P-labeled and gel purified before use as described below. Positive λgt11 cDNA clones were subjected to two further rounds of screening to obtain pure cDNA clones.

For phage dot blots, purified λgt11 phage were dotted onto bacterial lawns and probed with ^{32}P-labeled oligonucleotides exactly as for the initial library screening.

DNA sequencing

*Eco*RI inserts from positive λgt11 cDNA clones were cloned into the pSP72 vector (Promega) and inserts were then sequenced in both directions by the dideoxy sequencing procedure using progressive oligonucleotide primers. Sequences were compared to the GenBank database. Five cDNA clones encoded dbpB and two clones encoded a variant of dbpA (dbpAv). The dbpB λgt11 and pSP72 clones are designated λB1, λB2, λB3, λB4 and λB5 and pB1, pB2, pB3, pB4 and pB5 respectively. The dbpAv clones are named λA1 and λA2 and pA1 and pA2.

Plasmid constructs

*Eco*RI inserts from pB5 and pA2 containing full-length dbpB and dbpAv coding sequences were cloned in sense orientation into expression vector pSG5 (Stratagene), with a modified polylinker, to generate pSGdbpB and pSGdbpAv respectively. To generate the dbpB insert, pB5 was partially digested with *Eco*RI, as the dbpB coding region has an internal *Eco*RI site. pSGdbpBdel1 was created by digesting pSGdbpB with *Nar*I, which removes the CSD and the C-terminal domain of dbpB, followed by religation. pSGdbpBdel1 contains the first 47 amino acids of dbpB. pGM-41 and pGM-43 have previously been described and contain respectively the –114 to –66 region and the –65 to –31 region of the human GM-CSF promoter in the pBLCAT2 reporter vector (11). pSV2CAT contains the SV40 early promoter on the chloramphenicol acetyltransferase (CAT) reporter gene (23).

Cell culture and transfections

Human embryonic lung fibrobasts (HEL; Commonwealth Serum Laboratories, Australia) were grown in DMEM and 10% fetal calf serum. These cells were used from passage 14 to 20 in all experiments. HEL fibroblasts were co-transfected with 15 µg of reporter constructs and 5 µg of expression plasmids using DEAE–dextran as described (11). 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 assays performed as described (11). Percentage [^{14}C]chloramphenicol conversion to acetylated forms via CAT activity in extracts was determined using phosphorimager analysis (Molecular Dynamics).

Oligonucleotide probe preparation

All oligonucleotides were synthesized on an Applied Biosystems model 381A DNA synthesizer. Full-length oligonucleotides were purified from non-denaturing polyacrylamide gels (22). Single-strand DNA probes for gel retardation asays or library screening were prepared by end-labeling coding (+) or non-coding (–) strand oligonucleotides with [γ-^{32}P]ATP and T4 polynucleotide kinase followed by gel purification.

Preparation of nuclear extracts and gel retardation analysis

Nuclear extracts from HUT78 T cells were prepared as previously described (24). Gel retardation assays were performed using 0.25 ng of single-strand ^{32}P-labeled probe in a 10 µl reaction mix of 0.5× TM (25,11) buffer containing 200 mM KCl, 0.4 µg poly(dI·dC) and 1.0 µg of crude HUT78 T cell nuclear extract. Reactions were incubated at room temperature for 20 min and analyzed on 12% non-denaturing polyacrylamide gels in 0.5× TBE (25). Competition with unlabeled single-strand oligonucleotides was performed by addition of nuclear extract and unlabeled probe, followed by immediate addition of the labeled single-strand probe.

RESULTS

Isolation of CSD cDNA clones that bind to single-strand NF-GMb binding site DNA

Previous studies demonstrated that HUT78 T cell nuclear extracts contained high levels of protein capable of forming NF-GMb complexes (11). We therefore screened a λgt11 cDNA expression library, made from HUT78 T cell RNA, for clones expressing proteins with NF-GMb binding activity. The library was screened with a single-strand oligonucleotide (2×GM–) containing a double copy of the non-coding (–) strand of the –114 to –79 region of the human GM-CSF promoter. The non-coding strand of the –114 to –79 region contains the two repeated NF-GMb binding sites (5′-CCTG-3′) (11). After three rounds of screening seven positive clones were isolated. Inserts from the cDNA clones were subcloned into pSP72 plasmid vectors and sequenced. All of the clones contained open reading frames encoding CSD proteins. CSD proteins, also known as Y-box proteins, represent an unusual family of factors which have been shown to bind single-strand DNA, double-strand DNA and RNA and to be involved in transcriptional activation and repression, mRNA packaging and translational regulation (26–28). CSD proteins are divided into three domains, the central domain representing the CSD domain, which is highly conserved across this family of proteins (26–28).

Five of these clones (λB1–λB5, Fig. 1) contained DNA sequences identical to the cDNA sequence reported for the human CSD protein called dbpB (29). These sequences are also similar, but not identical, to the first reported sequence for human YB-1 (30). The differences in the YB-1 sequence are presumably due to sequence error and it is generally assumed that human dbpB and YB-1 are identical (31,32). The λB5 clone contains the full dbpB coding region and encodes a protein of 324 amino acids.

The remaining two clones (λA1 and λA2, Fig. 1) contained sequences similar to that reported for the cDNA encoding another CSD protein, dbpA (29). There are nine differences between the λA2 and the reported dbpA DNA sequences (Fig. 1). The differences in DNA sequence between the reported dbpA cDNA and those of λA1 and λA2 give rise to a different amino acid sequence. This variant protein sequence will be called dbpAv. The λA2 clone encodes the full dbpAv protein sequence. There are four amino acid changes (Ala75 of dbpA replaced by Thr; Pro340-Ser341-Ser342 of dbpA replaced by Arg-Pro-Pro) and an additional 30 C-terminal amino acids in the dbpAv protein (λA2) relative to dbpA. There are no amino acid changes between dbpA and dbpAv within the highly conserved CSD domain. The encoded dbpAv and dbpA proteins are respectively 372 and 342 amino acids in length. The extended sequence in dbpAv can be aligned with the C-terminal end of dbpB and terminates with the highly conserved Ala, Glu amino acid pair observed in most CSD proteins (27,33). Given the number of base changes between dbpA and dbpAv cDNA sequences we consider that the differences observed are real and not due simply to sequence error.

Proteins expressed from CSD cDNA clones bind to the repeated NF-GMb binding sites

To determine the binding specificity of the proteins expressed from dbpAv and dbpB cDNA clones, the clones were screened with a panel of probes in phage dot blots (Fig. 2). The clones were first screened with wild-type (GM) or mutant (GMm19, m21, m23 or m25) coding (+) or non-coding (–) single-strand oligonucleotides

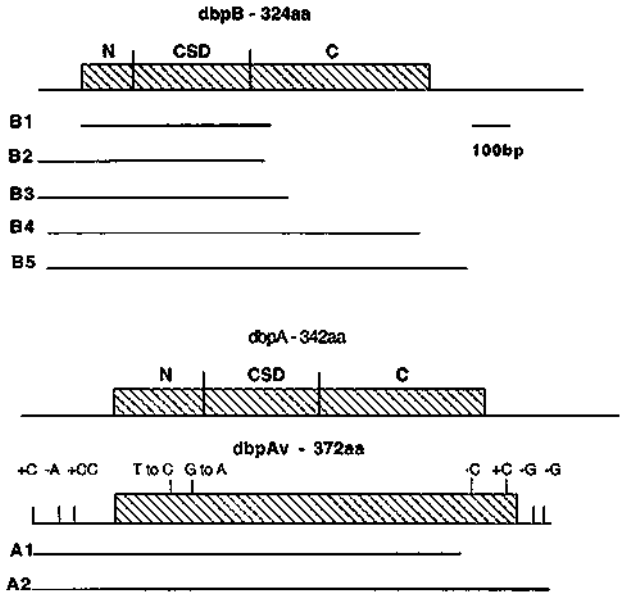


Figure 1. λgt11 cDNA clones expressing CSD proteins bind to the GM-CSF NF-GMb single-strand DNA binding site oligonucleotide. Seven cDNA expression clones were isolated from a λgt11 HUT78 T cell library using an NF-GMb binding site single-strand oligonucleotide (2×GM–) containing two copies of the –114 to –79 region of the GM-CSF promoter. Five clones (λB1, B2, B3, B4 and B5) contained sequence identical to dbpB. The locations of clone sequences relative to that published for dbpB (top of diagram) are shown. The location of the dbpB protein coding region is indicated by a hatched box. N-terminal (N), C-terminal (C) and CSD domains are marked. Two clones were similar to dbpA (λA1 and A2) and are named dbpAv. The locations of sequences in dbpAv clones relative to that published for dbpA are shown. The changes in the dbpAv DNA sequence relative to the dbpA sequence are indicated. From 5′ to 3′, a C is inserted between bases 39 and 40 of the dbpA sequence, an A at base 62 of the dbpA is deleted, CC is inserted between bases 85 and 86, T at base 396 of dbpA is changed to a C, G at base 433 is changed to an A, a C at base 1229 is deleted, a C is inserted between bases 1322 and 1323 and G residues are deleted at bases 1367 and 1381. Protein domains are marked on dbpA as above. The CSD domain represents the region highly conserved between CSD proteins (28,33).

covering the NF-GMb binding sites in the –114 to –79 region of the GM-CSF promoter (Fig. 2a). As expected, all cDNA clones expressed protein that bound to the screening probe (2×GM–; Fig. 2a). No binding or only a relatively small amount of binding was observed to the DNA strand complementary to the screening probe (2×GM+). As previously reported for binding of NF-GMb from fibroblast and HUT78 T cell nuclear extracts (11), protein expressed from all the cDNA clones bound to the wild-type GM (–114 to –79) non-coding (–) strand but did not bind to the coding (+) strand. We observed that the binding of protein from dbpB clones was consistently stronger than the binding of dbpAv protein to the GM– oligonucleotide. Consistent with NF-GMb binding activity, binding of protein from the dbpB and dbpAv clones was reduced or abolished by mutation of one or other of the NF-GMb binding sites (GMm19 or m21) and completely abolished when both sites were mutated (GMm23). Binding was not reduced by the irrelevant GMm25 mutation and, as we have previously observed for NF-GMb binding (11, unpublished data), the GMm25 mutant resulted in an increase in binding. This mutant increases the C content of the GM– sequence. As CSD proteins can have a preference for CT-rich sequences (28), this may explain the increased binding to the GMm25 mutant.

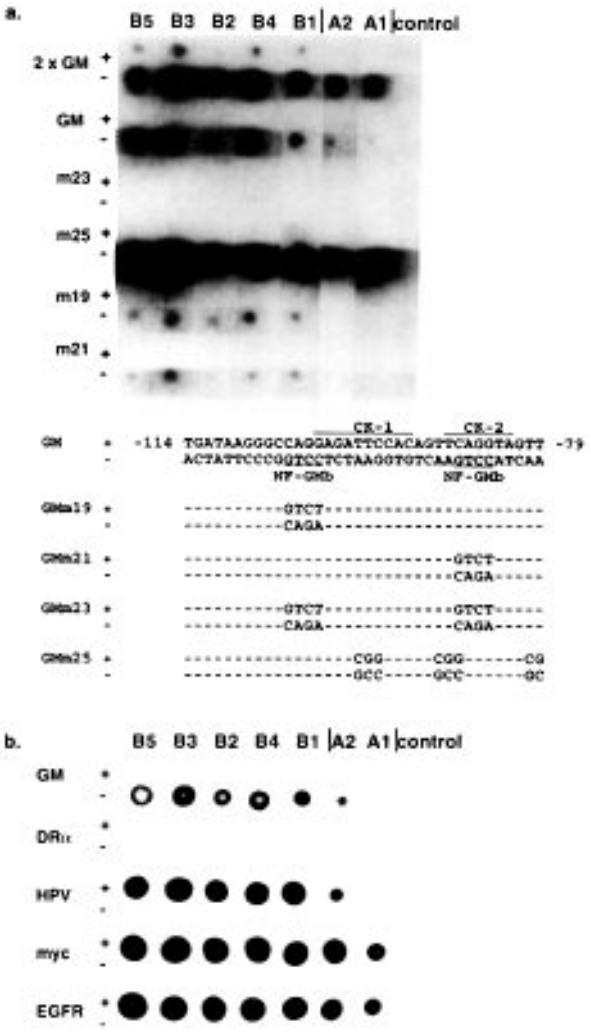


Figure 2. Protein from CSD cDNA expression clones bind to the single-strand GM-CSF NF-GMb binding sites and to single-strand CSD binding sites. (**a**) λgt11 expression clones (Fig. 1) were screened in phage dot blots with the ^{32}P-labeled library screening probe (2×GM–), with its complementary strand (2×GM+) and with coding (+) and non-coding (–) GM and mutant GM oligonucleotides. The sequences of the coding (+) and non-coding (–) strand single-strand GM oligonucleotides, containing the –114 to –79 region of the human GM-CSF promoter, are shown below the dot blot. The repeated NF-GMb binding sites (5′-CCTG-3′) on the non-coding strand are indicated. Sequences of mutant GM oligonucleotides are also shown. Only those bases that differ from the wild-type sequence are given. (**b**) Screening of expression clones with coding (+) and non-coding (–) strand GM-CSF (GM), HLA DRα Y-box region (DRα; 30) and CSD protein binding site oligonucleotides from the HPV 18 enhancer (HPV; 32), c-myc NSE (myc; 34) and EGF receptor NSE (EGFR; 34).

The proteins expressed from CSD cDNA clones were next screened with single-strand oligonucleotides previously reported to bind CSD proteins (Fig. 2b). The oligonucleotides represented the coding (+) and non-coding (–) strands of sequences from the HPV 18 enhancer (HPV; 32), the c-*myc* nuclease-sensitive element (NSE) (myc; 34) and the EGF receptor NSE (EGFR; 34). The HPV coding (+) sequence has been shown to bind human YB-1 (dbpB; 32). The myc coding (+) sequence binds dbpB and a human CSD protein called NSEP-1 (34,35) and the EGFR coding (+) sequence binds NSEP-1 (34). The coding (+) CSD binding strands are all CT-rich in nature. Consistent with these observations, the dbpAv and dbpB proteins expressed from cDNA clones bound specifically to the HPV+, myc+ and EGFR+ single-strand oligonucleotides, demonstrating no observable binding to the complementary non-coding (–) GA-rich strands. dbpB proteins bound all CSD oligonucleotides with similar affinity, whereas dbpAv protein had the highest affinity for the most highly CT-rich myc+ and EGFR+ oligonucleotides.

The expressed cDNA clones were also screened with the coding (+) and non-coding (–) strands of the Y-box motif (5′-CTGATTGGCCAA-3′) from the HLA DRα promoter (DRα; 30). This sequence was initially reported to bind YB-1 (dbpB) (30). There have been subsequent conflicting reports as to the ability of YB-1 (dbpB) to bind to Y-box sequences in genomic genes of higher organisms in either double-strand or single-strand form (33–38). In addition, NSEP-1 does not bind to a double-strand or single-strand Y-box sequence (34) and dbpA was found not to bind to a double-strand Y-box motif of the MHC gene I-Aβ (37). Consistent with these later observations, we find that dbpAv and dbpB expressed protein cannot bind to single-strand HLA DRα Y-box (DRα) coding (+) or non-coding (–) sequences (Fig. 2b).

The above results demonstrate that protein expressed from the dbpB and dbpAv cDNA clones have the appropriate binding characteristics previously described for CSD proteins and that they can bind to the GM-CSF promoter with the same specificity as that observed for nuclear NF-GMb.

CSD proteins are components of the nuclear NF-GMb complex

Given the above results, nuclear NF-GMb from HUT78 T cells was analyzed for its binding to the single-strand CT-rich CSD oligonucleotides in gel retardation assays. These assays will also detect any binding of the NF-GMc complex, which appears to be a component of the NF-GMb complex as determined from its binding characteristics and apparent protein content (11), as discussed above. In Figure 3a it can be seen that NF-GMb/c-like complexes bind to the HPV+ and EGFR+ sequences and an NF-GMc-like complex binds to the myc+ sequence. No binding was observed to the HLA DRα Y-box sequences (DRα), even on long exposure. Consistently these apparent NF-GMb/c complexes on the ^{32}P-labeled HPV+, myc+ and EGFR+ CSD binding site sequences were competed for by the unlabeled GM– oligonucleotide, whereas the extent of competition by GMm23– (NF-GMb binding site mutant) was considerably less (Fig. 3b). Competition was approximately five times greater with GM– than with GMm23–. Conversely NF-GMb/c complexes on the GM– oligonucleotide were competed for with the CSD binding site oligonucleotide HPV+ but not with a non-specific oligonucleotide (Fig. 3c). An additional complex marked x (Fig. 3a–c) was also seen in gel retardations. The nature of this complex is unknown, but we have demonstrated that it does not contact the repeated 5′-CCTG-3′ NF-GMb binding sites of the GM-CSF promoter (unpublished data). These data demonstrate that nuclear NF-GMb/c shows the same binding specificity for CSD oligonucleotides as the expressed CSD proteins. Consistently we also observed that NF-GMb/c complex formation on GM– or HPV+ was reduced by inclusion of a polyclonal antibody to a *Xenopus* CSD protein (a gift from A.Wolffe; data not shown). These data, taken together, indicate that CSD proteins are components of the nuclear NF-GMb/c complex.

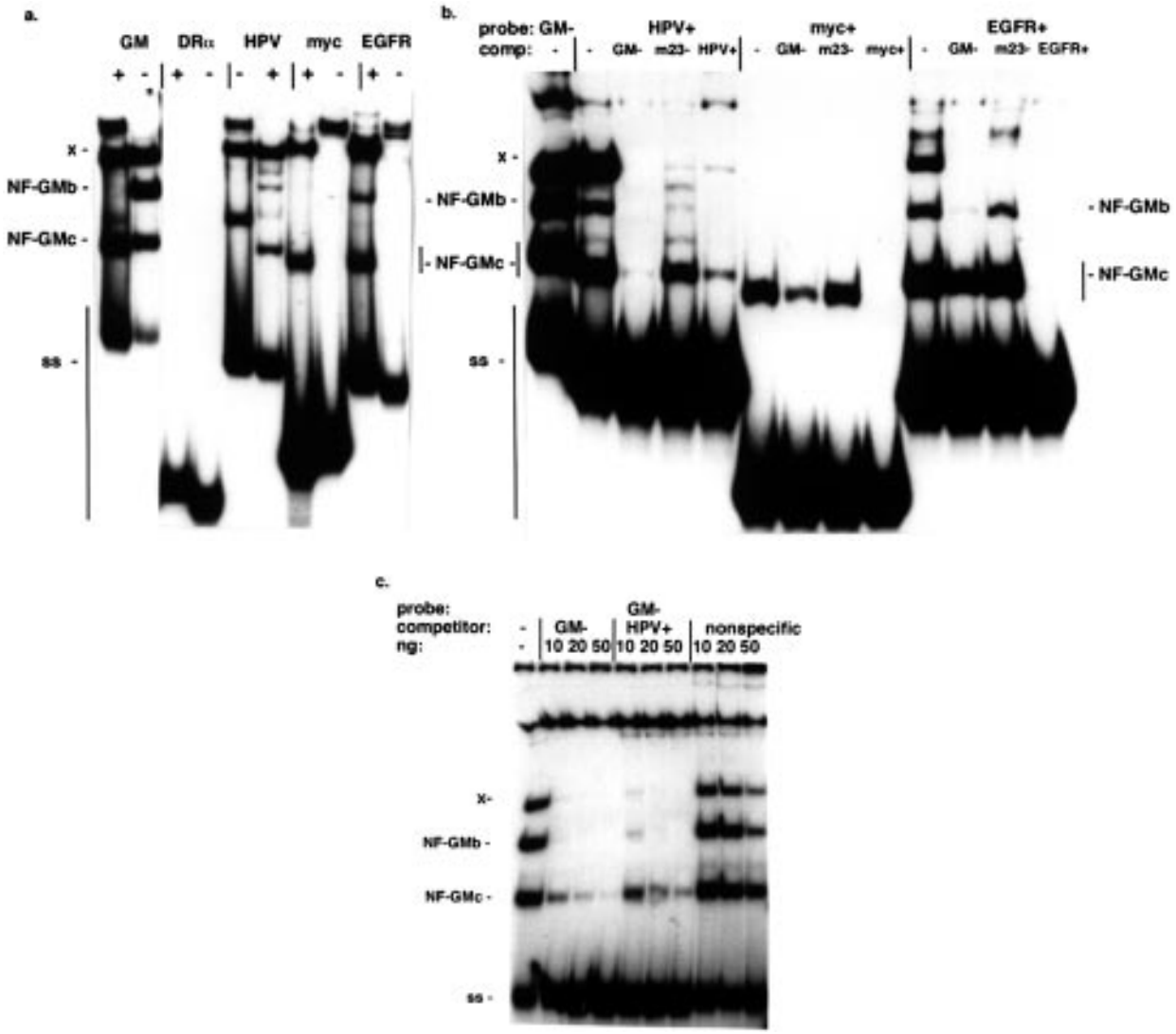
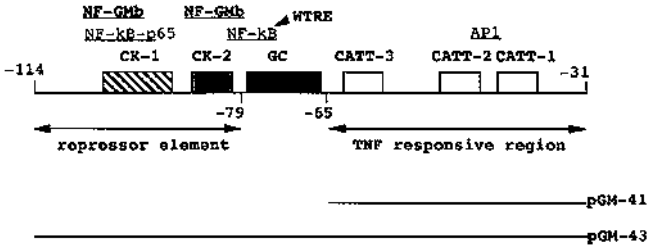


Figure 3. The NF-GMb complex contains CSD proteins. (**a**) Crude nuclear extracts from HUT78 T cells were analyzed in gel retardation asssays for binding to ^{32}P-labeled GM, Y-box (DRα) and CSD binding site (HPV, myc and EGFR) coding (+) and non-coding (–) strand single-strand oligonucleotides (Fig. 2). NF-GMb and NF-GMc complexes (formed on GM–, HPV+, myc+ and EGFR+ probes) are indicated and free single-strand probes are marked. The x indicates an as yet uncharacterized complex. (**b**) HUT78 T cell nuclear extracts were bound to ^{32}P-labeled CSD binding site HPV+, myc+ and EGFR+ oligonucleotides (probes) in the presence of 5 ng unlabeled GM–, GMm23– or self (HPV+, myc+ or EGFR+) competitor oligonucleotides (comp). (**c**) HUT78 T cell nuclear extracts were bound simultaneously to ^{32}P-labeled GM– oligonucleotide and varying amounts of unlabeled GM–, HPV+ and a non-specific sequence (5′-TCGAGAAGCTTCTGCAGTC-GACCCGGGAGCTCATTGGCGA-3′). Tracks with no competitor are marked –.

Repression of GM-CSF promoter sequences by over-expression of dbpAv and dbpB

Since we have shown in a previous report (11) that the NF-GMb/CSD binding sites were involved in repression of the –65 to –31 region of the GM-CSF promoter, we wished to determine the effect of overexpression of the CSD proteins on GM-CSF promoter function. To do this the pGM-41 (–65 to –31) and pGM-43 (–114 to –31) reporter constructs were co-transfected with the dbpAv (pSGdbpAv) and dbpB (pSGdbpB) expression clones into HEL fibroblasts and treated with or without TNF-α (Fig. 4). Consistent with the suggested function of NF-GMb/CSD, overexpression of both full-length dbpAv and dbpB repressed the 3-fold TNF-α-inducible activity from the pGM-43 construct, containing NF-GMb binding sites, by 67 and 60% respectively. Surprisingly, overexpression also repressed expression from the pGM-41 reporter construct. We are not aware of any NF-GMb/CSD binding sites in the –65 to –31 region (unpublished data). The TNF-α-inducible expression was reduced by 76 and 70% respectively by dbpAv and dbpB and the uninduced expression was reduced by 50% with both proteins. These results suggest that NF-GMb/CSD factors can also function in the absence of apparent binding sites.

The repressive effects discussed above were not however observed when either (i) pGM-43 and pGM-41 were co-transfected with pSGdbpBdel1, containing only the N-terminal sequences of dbpB, or (ii) when full-length dbpAv and dbpB constructs were co-transfected with a heterologous SV40 early promoter construct, pSV2CAT. As shown in Figure 4, pSGdbpBdel1 had little effect on pGM-43 expression and activated both pGM-41-induced (33%) and uninduced (50%) expression. dbpAv had little effect



constructs		-TNF	+TNF
pGM-43	+ pSG5	0.8	3.0
	+ pSGdbpAv	0.7	1.0
	+ pSGdbpB	0.9	1.2
	+ pSGdbpBdel1	1.0	3.3
pGM-41	+ pSG5	2.0	15.8
	+ pSGdbpAv	1.0	3.8
	+ pSGdbpB	1.0	4.7
	+ pSGdbpBdel1	3.0	21.0
pSV2CAT	+ pSG5	13.2	20.7
	+ pSGdbpAv	11.3	21.9
	+ pSGdbpB	10.6	15.9

Figure 4. Overexpression of dbpAv and dbpB results in repression of the GM-CSF promoter. A map of the GM-CSF proximal promoter is shown at the top of the diagram. Conserved elements are marked with boxes and the locations of transcription factor binding sites are marked (11). The TNF-α-responsive region and the repressor element containing NF-GMb/CSD binding sites are marked. The weakly TNF-α-responsive 3′ NF-κB element is marked WTRE. The relative location of pGM-41 and pGM-43 inserts are also shown. pGM-41, pGM-43 and pSV2CAT were co-transfected into HEL fibroblasts with the empty pSG5 expression vector, with expression clones containing full-length dbpAv (pSGdbpAv) and dbpB (pSGdbpB) sequences or with an expression clone containing the N-terminal sequences of dbpB (pSGdbpBdel1). Cells were treated with or without TNF-α. CAT activities (averages of at least three experiments) are given relative to untreated pBLCAT2 co-transfected with pSG5, which is given a value of 1.0. Given values have SE < 20%.

on induced SV40 promoter expression and a slight repressive effect (23%) was observed with dbpB (Fig. 4). As there are no reported studies on the function of CSD protein N-terminal domains, the reason for the activation effect of pSGdbpBdel1 is at present unclear. Given these results, however, it is clear that the high levels of repression of the GM-CSF promoter by full-length dbpAv and dbpB represent a specific repression of the GM-CSF promoter and are not just due to toxic affects of protein overexpression. It has also been reported that *Xenopus* CSD proteins can affect translation (27,28). The minor effects of dbpAv and dbpB overexpression on pSV2CAT expression rules out a major general effect on CAT reporter protein levels due to changes in translation.

DISCUSSION

We have cloned human cDNAs encoding proteins that bind to a single-strand DNA NF-GMb binding site probe derived from a novel repressor element in the human GM-CSF promoter (11). The cDNAs code for the CSD proteins dbpAv and dbpB. The dbpB sequence has previously been reported (29), while the dbpAv sequence is a new variant of the reported human dbpA sequence (29).

CSD proteins are characterized by a central domain of ~100 amino acids (26–28) which is highly conserved throughout evolution and is found to be 43% identical to bacterial cold shock proteins (26). Eukaryotic CSD proteins include dbpB (YB-1 and EF1a) (29–33,35,36,39–43), dbpA (29,31,35,44), NSEP-1 (34), FRGY1 and FRGY2 (45). In addition to binding single-strand DNA, CSD proteins can also bind double-strand DNA and RNA; the CSD domain is necessary for all these binding activities (31–35,46–48). By virtue of these varied nucleotide binding activities, CSD proteins have been shown to be involved in transcriptional activation and repression, mRNA packaging and translational regulation (27,28,36,37,39). The changes in protein sequence between dbpA and dbpAv are outside the CSD domain, resulting in a single amino acid substitution in the N-terminal region and a C-terminal extension on dbpAv. The functions of CSD protein N-terminal regions and the most C-terminal portions of these proteins have yet to be determined.

As for nuclear NF-GMb, dbpAv and dbpB expressed from cDNA clones required the repeated 5′-CCTG-3′ sequences on the non-coding (–) strand of the –114 to –79 GM-CSF promoter region for binding. Conversely, NF-GMb and the related complex NF-GMc bind to the same set of defined single-strand DNA CSD binding sites as the expressed CSD proteins. Competition and antibody assays indicated that CSD proteins are components of the nuclear NF-GMb/c complex. Accordingly, the identification of a 42.5 kDa protein in the NF-GMb complex, by UV-crosslinking (11), is consistent with the expected size for dbpAv (41 kDa) as deduced from the DNA sequence and with the observed size for CSD proteins detected by antibodies in HeLa and Raji cells (32,38).

Xenopus and bacterial CSD proteins (26,28,49) can bind a motif called a Y-box sequence (5′-CTGATTGGCCAA-3′). Mammalian/avian CSD proteins can also bind Y-box sequences in certain viral promoters (36,39,41). Mammalian/avian CSD proteins generally, however, have a preference for CT-rich sequences and their binding to Y-box sequences in genomic genes is controversial (28,34,35). Consistently we observed NF-GMb/CSD binding to three CT-rich single-strand DNA CSD binding sites, but not to a Y-box element. A common feature of the three CT-rich sequences investigated is the presence of repeated CT pairs (32,34). Six CT pairs can also be found in the GM-CSF promoter oligonucleotide (GM–), two of these in the repeated 5′-CCTG-3′ sequences involved in NF-GMb/CSD binding. We have also identified a similar repeated sequence in the G-CSF promoter that acts as a CSD binding site (unpublished data) and have reported potential NF-GMb (CSD) binding sites in the repressor elements of a number of other immune function genes (11).

We previously demonstrated that the –65 to –31 region of the GM-CSF promoter is TNFα responsive and that addition of the –114 to –66 region resulted in partial repression of this response (11). Mutation of the NF-GMb/CSD binding sites resulted in relief of this repression, indicating that the binding of NF-GMb/CSD factors was required for repressor element function. The binding of NF-GMb to the repressor element also appeared to prevent transcription factor action across the –114 to –66 region. The potential repressive action of NF-GMb factors is now confirmed here by our observation that overexpression of the CSD proteins dbpAv and dbpB results in near complete repression of the remaining TNF-α-inducible activity of the –114 to –31 construct (pGM-43). Repression of transcription by CSD proteins has also been reported for the HLA-DRα and I-Aβ genes and CSD binding sites have been located in a repressor element of the γ-globin genes (35,37,50). As NF-GMb (CSD) binding to the GM-CSF promoter is single-strand specific (11), we previously proposed that binding to single-strand DNA will affect

local DNA structure, preventing the binding of double-strand DNA-specific activating factors across the repressor element and the downstream –65 to –31 region. A similar mechanism of action has recently been suggested for the action of CSD proteins on the MHC class II DRα promoter and γ-globin genes (35,38). Consistently, single-strand regions have been detected in CSD binding sites *in vitro*, but have not yet been examined *in vivo* (34,35,38).

Transcriptional regulation by CSD factors may also be brought about via protein–protein interactions, as dbpA has recently been shown to form a complex with a subunit of the positive NF-Y transcription factor resulting in transcriptional repression of MHC class II genes (37). This complex formation occurs in the absence of a CSD binding site. Similarly, we observed here that overexpression of dbpAv and dbpB could directly repress the –65 to –31 region in the absence of the upstream NF-GMb/CSD binding site repressor element. We have not detected any NF-GMb/CSD binding sites in the –65 to –31 region (unpublished data). Mutation across the –65 to –31 region alone does not reveal any repressor elements, indicating that direct repression of this region does not involve a DNA sequence–protein interaction (unpublished data).

The relative contribution of repression of the GM-CSF promoter by the upstream repressor element (–114 to –66) and the direct action of CSD factors on the –65 to –31 region is difficult to estimate. It is of interest however that the addition of the upstream repressor element results in greater repression of the –65 to –31 region than overexpression of dbpAv and dbpB. The amount of repression by the –114 to –66 region is even greater when the most 3′ NF-κB site (Fig. 4), which is a weak activation site within this region, is mutated (11). It is possible that the binding of CSD factors to the –114 to –66 repressor element is the primary means of repression and that this is backed up by a second mechanism of repression acting directly on the –65 to –31 region. Such a dual mechanism of repression by CSD factors should ensure that there is no inappropriate expression of the endogenous GM-CSF promoter.

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