Regulation of *GM-CSF* Gene Transcription by Core-binding Factor[1]

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Abstract

***GM-CSF* gene activation in T cells is known to involve the transcription factors nuclear factor-κB, AP-1, NFAT, and Sp1. Here we demonstrate that the human GM-CSF promoter and enhancer also encompass binding sites for core-binding factor (CBF). Significantly, the CBF sites are in each case contained within the minimum essential core regions required for inducible activation of transcription. Furthermore, these core regions of the enhancer and promoter each encompass closely linked binding sites for CBF, AP-1, and NFATp. The GM-CSF promoter CBF site TGTGGTCA is located 51 bp upstream of the transcription start site and also overlaps a YY-1 binding site. A 2-bp mutation within the CBF site resulted in a 2–3-fold decrease in the activities of both a 69-bp proximal promoter fragment and a 627-bp full-length promoter fragment. Stepwise deletions into the proximal promoter also revealed that the CBF site, but not the YY-1 site, was required for efficient induction of transcriptional activation. The *AML1* and *CBFβ* genes that encode CBF each have the ability to influence cell growth and differentiation and have been implicated as proto-oncogenes in acute myeloid leukemia. This study adds GM-CSF to a growing list of cytokines and receptors that are regulated by CBF and which control the growth, differentiation, and activation of hemopoietic cells. The *GM-CSF* locus may represent one of several target genes that are dysregulated in acute myeloid leukemia.**

Introduction

GM-CSF[3] is a cytokine that regulates the proliferation, differentiation, and function of cells of the granulocyte, macrophage, and eosinophil lineages (1). GM-CSF is induced in T cells by TCR stimulation and in myeloid cells, endothelial cells, and fibroblasts by pro-inflammatory cytokines such as IL-1 (1, 2). GM-CSF is also produced either constitutively or in response to agents such as IL-1 in a high proportion of AMLs (3). Although GM-CSF acts as a growth factor for some AMLs, in other cases it may lead to differentiation and extinction of leukemic cells (4, 5).

The human *GM-CSF* locus is regulated by a promoter (6–8) and an upstream enhancer (9–11) that both respond to TCR signals. These signals induce highly cooperative binding of NFATp/c and AP-1 to several sites in the enhancer and moderate binding of these factors to overlapping sites in the proximal region (−33 to −47) of the promoter. The distal region of the promoter (−70 to −103) associates with members of the nuclear factor-κB/Rel (7, 12) and Sp1 (13) families of transcriptional activators. Although the intervening segment of the promoter (−48 to −69) is activated by additional unknown factors (14–16), it can also be repressed by the transcription factor YY-1 (15). The highly conserved region of the promoter extends 116 bp upstream of the transcription start site and is sufficient to support maximum promoter activity.[4]

We sought additional transcription factors that may either determine the cell specificity of GM-CSF expression or be involved in dysregulated GM-CSF expression in AML. CBF is one such factor that required investigation because it functions in cell types that express GM-CSF (17–19) and its genes are frequently disrupted in AML (20–22). CBF is constitutively expressed in T cells and myeloid cells and binds to DNA as a complex of two proteins, CBFα and CBFβ. Genes known to be regulated by CBF include cytokines and receptors such as IL-3, the TCR, and the CSF-1 receptor (17–19). In retroviruses, CBF motifs are determinants of T cell tropism (17). The *AML1* gene, which encodes CBFα, and the CBFβ gene have both been identified as common chromosomal translocation breakpoints in AML, thus implicating them as a proto-oncogenes (20–22). *AML1* gene translocations convert CBF into a dominant repressor (20, 21).

We searched the GM-CSF promoter and enhancer for CBF-like motifs and in each case identified CBF-binding sites in essential core regions. These findings have implications regarding the role of CBF in T cells and in AML.

Results

The GM-CSF Proximal Promoter Encompasses a CBF Binding Site. To search for transcription factor binding sites that might regulate GM-CSF expression in both T cells and myeloid cells, we screened the GM-CSF promoter for CBF

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[3] The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; AML, acute myeloid leukemia; TCR, T cell receptor; CBF, core-binding factor; PMA, phorbol 12-myristate 13-acetate.

[4] P. Cockerill and C. Osborne, unpublished data.

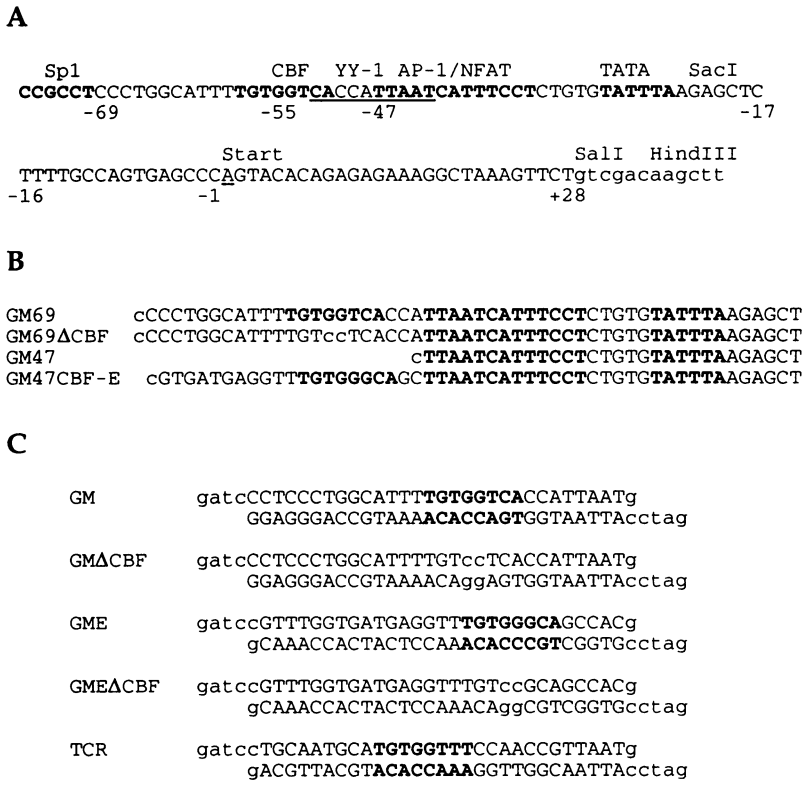

Fig. 1. GM-CSF promoter sequences and oligonucleotides used in plasmids and binding assays. Native GM-CSF promoter sequences are shown in *uppercase*, while altered bases and cloning sites are shown in *lowercase*. Transcription factor binding sites are either *underlined* or shown in *bold* typeface. *A*, sequence of the human GM-CSF promoter depicting transcription factor binding sites and positions in the sequence relative to the transcription start site (*underlined*). The sequence includes the segment of the 5′ untranslated region and downstream cloning sites that were incorporated into each plasmid used in this study. *B*, oligonucleotides incorporated into the luciferase reporter gene plasmids pGM69, pGM69ΔCBF, pGM47, and pGM47CBF-E. *C*, GM-CSF promoter and enhancer and TCR δE3 CBF-binding oligonucleotides.

motifs. Within the vicinity of a YY-1 binding site, we identified the sequence TGTGGTCA (Fig. 1*A*) that is identical to the CBF site conserved among mammalian type C retroviruses (24). To test the properties of this motif in gel electrophoretic mobility shift assays, we designed a probe that encompassed the YY-1 and CBF-like sequences but not the downstream AP-1 motif or the upstream Sp1 site (Fig. 1*C, GM*). For a control, we used the well-characterized TCR-δE3 CBF site (Fig. 1*C, TCR*; Ref. 17).

The GM-CSF promoter GM probe had binding properties similar to the TCR CBF probe. Hence, the GM-CSF probe formed specific complexes of the same mobility as the TCR probe (Fig. 2, *Lanes 1* and *8*). The GM element functioned as a specific competitor of CBF binding to the GM-CSF and TCR probes and, in turn, the TCR element partially suppressed CBF binding to the GM-CSF probe. The GM-CSF promoter may represent a relatively high affinity CBF-binding site because the TCR probe was a less effective competitor than the GM probe. Complexes formed with the GM-CSF probe were formally identified as CBF complexes on the basis that they were eliminated by antisera raised against either the CBFα or CBFβ components of CBF but not by an unrelated antisera. This conclusion was further supported by the detection of faint supershifted bands (Fig. 2, *SF→, Lanes 5* and *6*) seen with both CBF antibodies but not with the control antibody. In common with other CBF sites (17, 18), the central GG sequence was essential for binding because a mutation of this sequence to CC eliminated formation of CBF complexes (Fig. 1*C, GMΔCBF*; Fig. 2, *Lane 12*). The GMΔCBF oligonucleotide also lacked the ability to efficiently inhibit CBF binding to either the GM-CSF or TCR probes (Fig. 2, *Lanes 3* and *10*). Curiously, we saw no evidence of YY-1 binding to the GM-CSF probe as the only other complex observed was a nonspecific one.

The GM-CSF Enhancer Core Also Encompasses a CBF Binding Site. Because *GM-CSF* gene induction involves both the promoter and the upstream enhancer, we screened the 716-bp enhancer sequence (9, 11) for CBF-like motifs. We identified the CBF-like motif TGTGGGCA (termed here as the GM450 element) just downstream from the GM420 NFAT enhancer element and within the previously defined inducible DNaseI hypersensitive site (9, 10). The GM420 element is the highest affinity NFATp/c-binding sequence in the enhancer, and its closely linked AP-1 and NFATp/c motifs represent the only functional transcription factor binding sites known to exist within the 160-bp essential core region (10). It is highly likely that at least one other transcription factor binding site resides within this core because it has an activity similar to that obtained with the full-length enhancer (10). Significantly, the CBF-like motif also lies within a conserved region of this

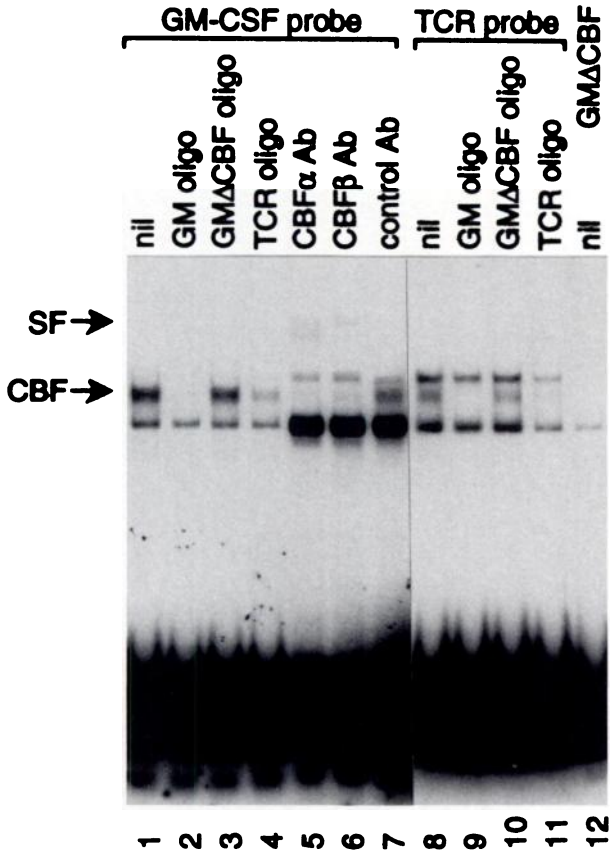

Fig. 2. Gel electrophoretic mobility shift assays of Jurkat T cell nuclear extracts with GM-CSF promoter and TCR δE3 CBF-binding oligonucleotides. Note that the antisera give rise to additional nonspecific bands above and below the CBF complex in *Lanes 5–7* and faint supershifted bands (*SF*) in *Lanes 5* and *6*.

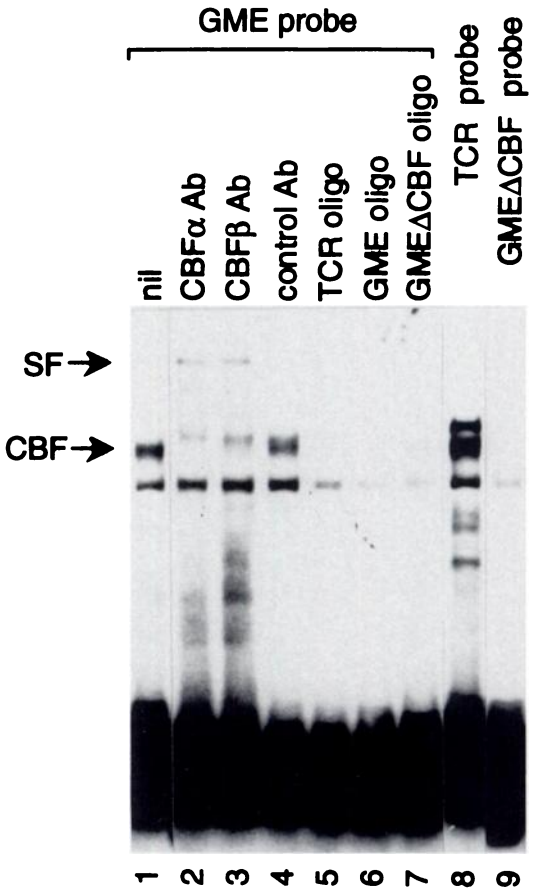

Fig. 3. Gel electrophoretic mobility shift assays of Jurkat T cell nuclear extracts with GM-CSF enhancer and TCR δE3 CBF-binding oligonucleotides. Note that the antisera give rise to additional supershifted bands (*SF*) in *Lanes 2* and *3*.

essential core (10, 11) and is within a region that is protected by nuclear factors in footprinting studies (10).

The GM450 motif was identified as a CBF-binding site by the same criteria used to define the CBF site in the promoter (Fig. 3). An oligonucleotide probe encompassing the GM450 motif (Figs. 1*C*, *GME*, and 3) associated with a CBF-like factor (Fig. 3, *Lanes 1* and *8*) that was specifically supershifted by CBFα and β antibodies (Fig. 3, *SF→*, *Lanes 2* and *3*). The CBF complex was completely blocked by either the TCR competitor or excess GME DNA (Fig. 3, *Lanes 5* and *6*) but was only partly blocked by a mutated GME competitor (Fig. 3, *Lane 7*). Furthermore, the GG to CC mutation within the GMEΔCBF probe was sufficient to eliminate CBF binding (Fig. 3, *Lane 9*). By comparison with the data in Fig. 2, the GME probe appeared to be a weaker CBF binding site than either the GM or the TCR probes. The GM-CSF enhancer CBF site overlaps the sequence TGAGGTTT that also bears some resemblance to CBF elements, but this sequence was unable to support detectable CBF complex formation with the GMEΔCBF probe (Fig. 3, *Lane 9*). This sequence may, however, function as a very weak CBF site and account for the partial competition of CBF binding seen in Fig. 3, *Lane 7*.

The CBF Sites Support Inducible GM-CSF Promoter Function. To examine the function of the CBF site in the promoter, we prepared luciferase reporter gene plasmids in which the GM-CSF proximal promoter was truncated either immediately after the AP-1 site at −47 (GM47), at the *Bst*EII site at −55 (GM55), just before the Sp1-like motif (13) at −69 (GM69), or at −627 to include all of the promoter (GM627; Figs. 1 and 4). The GM47 construct has a truncated YY-1 motif, whereas the GM55 construct has lost the ability to bind CBF (data not shown). Each GM-CSF promoter/luciferase reporter gene plasmid was transiently transfected into Jurkat T cells. Because GM-CSF expression is highly inducible, we measured luciferase expression in transfected cells that were either unstimulated or stimulated for 9 h in the presence of PMA and A23187 (Fig. 4, *P/I*). This combination of phorbol ester and calcium ionophore mimics signals that normally originate from the TCR.

The pGM47 and pGM55 plasmids, which encompass the AP-1 and NFAT sites but not the CBF motif, were very poorly induced, and the addition of the YY-1-like motif had no influence upon promoter activity. The inclusion of the CBF site increased the inducible activity of the proximal promoter 2-fold while having little effect on its constitutive activity

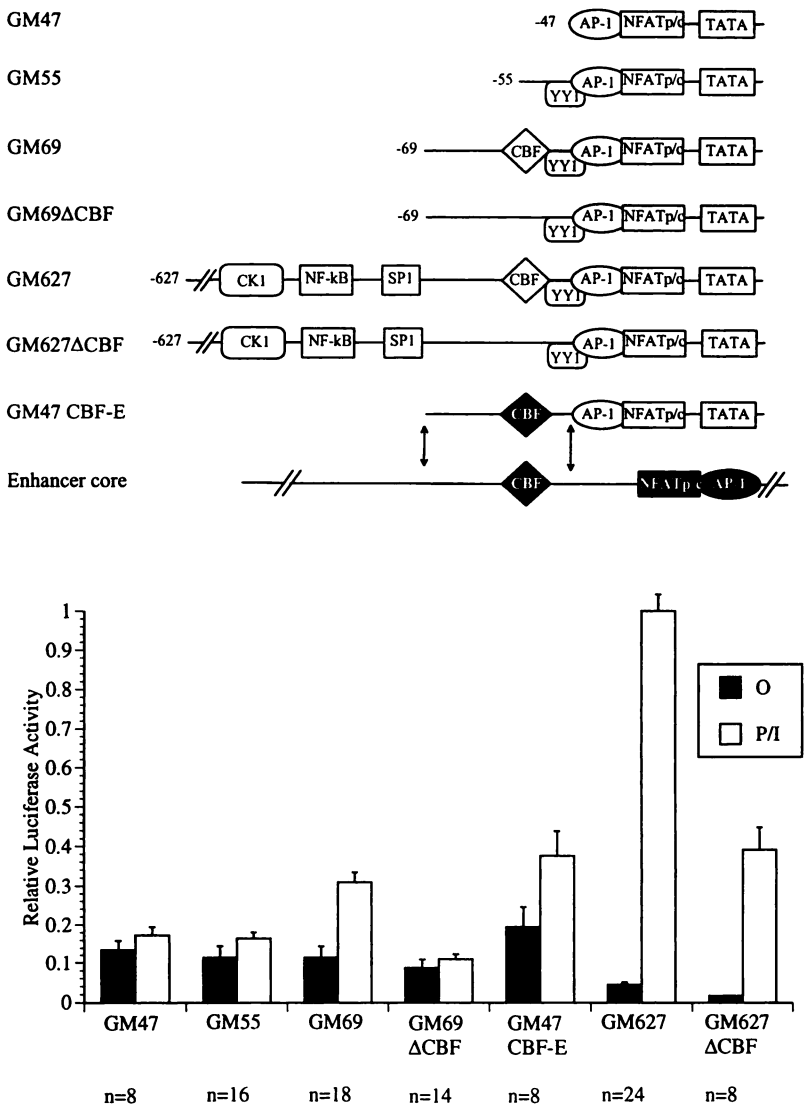

Fig. 4. Transient transfection assays of GM-CSF promoter/luciferase reporter gene plasmids in Jurkat T cells. Several transfections were performed on each of two independent clones of each plasmid, with the total number of assays indicated by *n*. Twenty-four h after transfection, cells were stimulated for 9 h with PMA and A23187 (*P/I*), and cells were harvested for luciferase assay. □, the mean of at least eight transfections, and the data are expressed relative to pGM627; *bars*, SE. ■, the mean of at least four unstimulated control transfections; *bars*, SE.

(pGM69; Fig. 4). This 69-bp proximal region of the promoter was sufficient to support a level of activity one-third of that obtained with the −627 fragment that encompasses the full-length promoter (pGM627). The pGM69 plasmid relied on an intact CBF binding site for its increased level of inducible activity as a 2-bp mutation within the CBF site reduced promoter activity by 3-fold (Fig. 4, *GM69ΔCBF*). The GM69ΔCBF plasmid carries the same 2-bp mutation as that present in the GMΔCBF probe that was sufficient to abolish CBF binding. The CBF site was also required for efficient activation of the full-length promoter as the same CBF site mutation introduced into the pGM627 plasmid resulted in a 2–3-fold decrease in activity (Fig. 4, *GM627ΔCBF*).

Similar to the promoter CBF site, the GM-CSF enhancer GM450 CBF binding site also sits in close proximity to AP-1 and NFATp/c motifs (Fig. 4). With the exception of the non-functional GM400 AP-1 motif, these three closely linked motifs are the only transcription factor binding sites known to exist within the 160-bp enhancer core (10). We, therefore, assessed the function of the GM450 motif by linking it directly upstream of the AP-1 and NFAT sites present in pGM47 (pGM47CBF-E). We again observed a doubling of the inducible activity of the promoter, indicating that the GM450 CBF motif is also a functional transcription activation element.

Discussion

The activity of the GM-CSF promoter appears to be governed by a cluster of NF-κB and Sp1 sites in the distal region

and closely associated CBF, AP-1, and NFATp/c sites in the proximal region. The proximal region of the promoter was found in this study to be able to support one-third of the activity of the full-length promoter, and its inducible activity was highly dependent upon an intact CBF binding site. At least three previous studies have demonstrated that the CBF region is required for promoter function (14–16), but these studies did not identify CBF as a factor binding in this region. A preliminary report from the laboratory of Dr. S. Nimer does, however, also indicate that CBF is an important regulator of the human GM-CSF promoter (25). The CBF site is highly conserved because the TGTGGTCA motif exists in both the mouse and human GM-CSF promoters (13). Indeed, a recent report has found that the mouse GM-CSF promoter is also regulated by CBF-like factors (26). It is unclear, however, why we have not detected YY-1 binding to the GM-CSF promoter, as has been reported by others (15). These differences may relate to binding conditions, the cells used, or the different choices of probes used for DNA binding. Because there is some evidence that the GM-CSF promoter is repressed by YY-1, we suggest that CBF and YY-1 represent an activator and a repressor competing for occupancy of the same site. Because the proposed YY-1 site also overlaps the adjacent AP-1 site (15), the balance may be tipped in favor of CBF binding upon induction of AP-1 via the TCR pathway. In contrast to a previous report (16), we have found no evidence for the existence of an inhibitory element upstream of the AP-1 site.

Interestingly, the CBF sites in the promoter and enhancer exist in a similar context. Both reside within regions defined as the minimum essential core required for significant inducible activity. In each case, these core regions comprise single closely linked binding sites for CBF, AP-1, and NFATp/c. We suggest, therefore, that these three sites cooperate as a functional unit in the GM-CSF promoter and enhancer. There is also some evidence to suggest that these factors may synergize via distinct mechanisms. We have found NFAT sites to be closely associated with DNaseI hypersensitive sites (10), and our preliminary evidence suggests that NFATp/c proteins can function as chromatin modifying factors.[5] CBF may also have a distinct role because it appears to be tightly associated with structures within nuclei (21). Our own preliminary immunohistochemistry using CBFα antisera with Jurkat cells revealed a speckled staining pattern reminiscent of transcriptionally active domains within the nuclear matrix.[6] Taken together, these observations may suggest a function for CBF as an intranuclear homing signal that allows other adjacent transcription factor binding sites to dock with the transcription apparatus within transcriptionally active nuclear domains.

GM-CSF is expressed in a variety of cell types, and it is likely that CBF regulates GM-CSF promoter function in myeloid cells and endothelial cells as well as in T cells. Previous studies in endothelial cells found the CBF-binding region to be required for efficient GM-CSF promoter activation (14).

CBF could also contribute to the autocrine expression of GM-CSF in the subset of AMLs that rely on GM-CSF for their growth. Paradoxically, however, *AML1* gene translocations lead to expression of a dominant repressor form of CBF (20, 21). This raises the intriguing possibility that there exists another subset of AMLs that avoid programmed differentiation by suppressing expression of differentiation factors such as GM-CSF, or their receptors. There are two lines of evidence that would support such a hypothesis: (*a*) there do exist AMLs that can be extinguished *in vivo* by administration of GM-CSF (5), and GM-CSF induces differentiation and clonal extinction of human HL60 leukemic cells in culture (4); and (*b*) there is evidence that GM-CSF can act as an autocrine differentiation factor (27). Thus, CBF has the potential to influence the growth and differentiation of myeloid cells by either activating or repressing GM-CSF expression.

Materials and Methods

Plasmid. Luciferase reporter gene plasmids were constructed by inserting human GM-CSF promoter fragments within the multiple cloning sites of pGL3-Basic (Promega). Promoter fragments originated from either oligonucleotides or pGMluc (12), and their sequences were verified as being the same as the reported human GM-CSF sequence (23). Fig. 1*A* shows the sequence of the proximal promoter and the 5′ untranslated region that is common to all of the plasmids created in this study. pGM627 was constructed by inserting a *Hin*dIII fragment of pGMluc into the *Hin*dIII site of pGL3-Basic and contains the full-length GM-CSF promoter (−627 to +28). pGM55 has the proximal promoter sequence truncated at −55 and was constructed by deleting the *Bst*EII fragment of pGM627 that encompasses the −627 to −56 segment of the promoter. pGM47, pGM69, pGM69ΔCBF, and pGM47CBF-E were constructed by deleting a *Sac*I fragment (−627 to −21) from pGM627 and inserting the oligonucleotides depicted in Fig. 1*B* in its place. pGM627ΔCBF was constructed by site-directed mutagenesis of the *Hin*dIII fragment of pGMluc in the plasmid pAlter and using an Altered Sites II kit, according to manufacturer's instructions (Promega) with the CBF mutant site oligonucleotide CCCTGGCATTTTGTCCTCACCATTAATC.

Gel Electrophoretic Mobility Shift Assays. Nuclear extracts were prepared from Jurkat T cells and assayed essentially as described previously (9, 10). Briefly, 4 μg of nuclear protein were incubated with 0.2 ng of probe DNA in the presence of 4 μg of poly(dI-dC) and 100 ng boiled sonicated herring DNA. Where indicated, 25 ng of oligonucleotide duplex competitor or 3 μl of antisera was included during the incubation. CBFα and CBFβ antisera were obtained from N. Speck (Dartmouth Medical School, Hanover, NH; Ref. 18). The unrelated control rabbit antisera was raised against the GM-CSF receptor.

Transfections and Luciferase Assays. Jurkat cells were transfected with 5 μg CsCl-purified plasmid DNA by electroporation, stimulated with 20 ng/ml PMA, and 1 μM calcium ionophore A23187 and assayed for luciferase, as described previously (11).

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