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Characterization of a Cell-Type-Restricted Negative Regulatory Activity of the Human Granulocyte-Macrophage Colony-Stimulating Factor Gene

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Human granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulates the proliferation and maturation of normal myeloid progenitor cells and can also stimulate the growth of acute myelogenous leukemia (AML) blasts. GM-CSF is not normally produced by resting cells but is expressed by a variety of activated cells including T lymphocytes, macrophages, and certain cytokine-stimulated fibroblasts and endothelial cells. Production of GM-CSF by cultured AML cells has been demonstrated, and GM-CSF expression by normal myeloid progenitors has been postulated to play a role in myelopoiesis. We have investigated the regulation of expression of GM-CSF in AML cell lines, and our results demonstrate the presence of a strong constitutive promoter element contained within 53 bp upstream of the cap site. We have also identified a negative regulatory element located immediately upstream of the positive regulatory element (within 69 bp of the cap site) that is active in AML cell lines but not T cells or K562 CML cells. Competition transfection and mobility shift studies demonstrate that this activity correlates with binding of a 45-kDa protein.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a glycoprotein cytokine with important roles in hematopoiesis and host defense (reviewed in reference 11). GM-CSF promotes the proliferation and maturation of myeloid progenitor cells (30) and enhances the functional activity of the mature myeloid effector cell pool (27, 32, 33). In vivo activity of GM-CSF has also been demonstrated, as numerous clinical trials have shown that GM-CSF stimulates a dose-dependent increase in the number of functional leukocytes (12, 31). GM-CSF is expressed by a variety of cells following activation by inflammatory signals or cytokines, consistent with a role in regulating hematopoiesis and immune function. Antigen- or mitogen-stimulated T lymphocytes (8, 35), activated macrophages (29), and certain interleukin 1 (IL-1)- or tumor necrosis factor-stimulated fibroblast and endothelial cells express GM-CSF (5, 18), as do activated mast cells (34).

Regulation of expression of GM-CSF in different cell types results from an interplay of transcriptional and posttranscriptional mechanisms. GM-CSF production by fibroblasts stimulated with IL-1 or tumor necrosis factor appears to be controlled posttranscriptionally (14, 21), and Shaw and Kamen have demonstrated that the AT-rich region at the 3′ end of the GM-CSF gene is important in determining mRNA stability (26). However, transcriptional control also plays a critical role in regulating GM-CSF expression in various cell types, including activated T cells. We have previously shown that sequences contained within 53 bp 5′ of the mRNA cap site are sufficient to direct inducible expression of a reporter gene in stimulated T-cell lines (8, 20, 22). This activity is dependent upon a repeat of the sequence CATT$^{A}_{T}$ present from nucleotides −48 to −37 (20). Miyatake et al. have confirmed this observation, using murine GM-CSF promoter sequences (17).

While expression of GM-CSF has not been detected in fresh blast cells from patients with acute myelogenous leukemia (AML), expression has been detected in AML blast cells in culture (36, 37). These cells express high-affinity receptors for GM-CSF (10, 13) and will proliferate in response to GM-CSF in vitro (2). Interestingly, IL-1-inducible expression of GM-CSF by the normal counterparts of these cells has also been described (3), and it has been suggested that these phenomena result in a prolongation and enhancement of the response of progenitor cells to primary differentiation signals (25). Alternatively, expression of GM-CSF by leukemic cells which are GM-CSF responsive suggests a potential autocrine loop in the generation or progression of myeloid leukemia (37).

In this study, we examine several elements of the GM-CSF promoter to determine the mechanisms controlling GM-CSF expression in AML cells. We define a regulatory model in which binding of an activating transcription factor complex is prevented by binding of a 45-kDa nuclear factor to inhibitory sequences which overlap the positively acting promoter element. The activity of the inhibitory element appears to be restricted to AML cells, as similar activity and competitive binding was not observed in other cell types.

MATERIALS AND METHODS

Transfection procedure. The human Jurkat T-cell line and the KG-1 (AML) and K562 (chronic myelogenous leukemia [CML]) leukemic cell lines were maintained in Iscove's modified Dulbecco's medium supplemented with 10% bovine serum, 1% glutamine, and penicillin plus streptomycin. Factor-dependent MO-7 cells (1) were maintained in Iscove's modified Dulbecco's medium supplemented with 20% bovine serum and ≥200 pM recombinant human GM-CSF. Electro-

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poration was performed by the method of Cann et al. (7); 5 × 10^6 cells were electroporated with 25 μg of plasmid DNA by using a Bio-Rad Gene Pulser, set at 250 V and 960 μF. Following electroporation, cells were resuspended in their initial culture medium for 24 h. Cells were harvested, and cell extracts were assayed for chloramphenicol acetyltransferase (CAT) activity, as described previously (20). Cotransfection with an internal control plasmid (the cytomegalovirus enhancer linked to the luciferase gene) demonstrated essentially equivalent transfection efficiency for all constructs. S1 nuclease protection with a probe containing 34 nucleotides (nt) of GM-CSF cDNA sequence, 25 nt of adjacent promoter sequence, and 24 nt of irrelevant DNA sequence confirmed correct initiation of transcription (28 to 30 nt downstream of the start of the TATA homology [8]) for the largest (−626CAT) and smallest (−53CAT) constructs (data not shown). For competition cotransfection studies, the amount of plasmid DNA transfected remained the same (25 μg), comprising 5 μg of −53CAT reporter plasmid and a total of 20 μg of competitor plasmids −69pUCΔCATT-1,-2 (or control plasmid −69pUCΔCATT-1,-2,-3) and pUC18 in different ratios to give a maximum molar ratio of 33.5 to 1, competitor to reporter.

Construction of plasmids. The methods used for constructing the parent GM-CSF–CAT recombinant plasmid, −626CAT (+pCSFp1); the 5′ deletion mutants −193CAT, −179CAT, −106CAT, and −53CAT; and the internal deletion mutant −626CATΔ-60/-39 have been described previously (8, 20, 22). The 5′ deletion mutant −336CAT was made by limited *Bal* 31 digestion of −626CAT, which was linearized by *Hin*dIII digestion 626 bp upstream of the GM-CSF cap site (Fig. 1A). Constructs −507CAT and −217CAT were made by double digesting construct −626CAT with *Hin*dIII and either *Ppu*MI or *Eco*NI (constructs −507CAT and −217CAT, respectively), treatment with the large (Klenow) fragment of DNA polymerase I, and religation. Construct −458CAT was made by cloning the *Sst*I fragment of −626CAT into *Hin*dIII-*Sst*I-digested −626CAT. Similarly, constructs −412CAT and −398 CAT were prepared by cloning the *Mbo*II-*Sst*I or *Ban*I-*Sst*I fragment of −626CAT, respectively, into *Hin*dIII-*Sst*I-digested −626CAT. Construct −626CATΔBKL was prepared by treating *Bst*EII-digested −626CAT with the large (Klenow) fragment of DNA polymerase I and subsequent religation. The −69CAT construct was created by synthesizing a double-stranded oligonucleotide which was cloned into the *Bam*HI and *Sst*I sites of pUC18 and then subcloned into *Hin*dIII-*Sst*I-digested −626CAT. −69CATΔ66, −69CATΔ60, and −69CATΔ55 were prepared by synthesizing complementary oligonucleotides and cloning directly into *Hin*dIII-*Sst*I-digested −626CAT. Constructs −626CATΔ-53/-49, −626CATΔCATT-1, −626CATΔCATT-2, and −626CATΔCATT-1,-2 were prepared as previously described (20). −53CATΔCATT-1 was prepared by *Hin*dIII-*Bst*EII digestion of −626CATΔCATT-1; single-stranded overhangs were filled in with Klenow and then by religation. Constructs −69pUCΔCATT-1,-2 and −69 pUCΔCATT-1,-2,-3 were prepared by synthesizing complementary oligonucleotides (sequence shown in Fig. 5) and cloning directly into *Bam*HI-*Sst*I-digested pUC18. The extent of all deletions and the presence of site-directed mutations for all constructs were determined by DNA sequencing by either dideoxy (24) or Maxam-Gilbert (15) sequencing methods.

Analysis of DNA-protein interactions. Electrophoretic mobility shift assay (EMSA) probes were prepared by synthesis of single-stranded oligonucleotides with an Applied Biosystems, Inc., synthesizer. The sequences of the oligonucleotides are as follows:

−52/−33, GATCTCACCAXXAATCAXXTCCTG
AGTGGXAATTAGXAAAGGACCTAG

−69/48, GATCCCTGGCAXXTXGTGGXCACC
AGGGACCGTAAAACACCAGTGGTC

ΔCATT-1-2, GATCTGGTCACaAgggATagaagCTTCTGGTCACaAgggATagaagCTTCTG
ACCAGTGtTcccTAtcttcGAAGACCAGTGtTgggTAtcttcGAAGACCTAG

−69Δ-1-2-3, GATCCCTGGatccTTGTGGTCACaAgggATagaagCTTGTG
GGACCtaggAACACCAGTGtTcccTAtcttcGAACACCTAG

CRE2, AGCTTTCACGTCATCACGTCACT
AAGTGCAGTAGTGCAGTGAGATC

X indicates substitution of bromodeoxyuridine for thymidine. Lowercase letters indicate regions of base substitution from wild-type sequences.

Complementary strands were annealed and end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Labeled probe was purified from free nucleotide by use of Push Columns (Stratagene). In order to enhance the efficiency of UV cross-linking, thymidine residues within the regulatory regions (see above) were substituted with bromodeoxyuridine during synthesis. These substitutions had no effect on the gel shift pattern but improved cross-linking by more than an order of magnitude (data not shown).

Crude KG-1 nuclear extract was fractionated with a 5-ml HiTrap heparin agarose column (Pharmacia-LKB). Protein was eluted with 10-ml steps containing 0.3, 0.5, 0.7, and 1.0 M KCl. EMSAs were performed by incubating 1 ng of ^{32}P-labeled oligonucleotide probe with 2 μg of heparin agarose-fractionated nuclear extract in the presence of 1 μg of poly(dI-dC), 500 ng of mutant competitor ΔCATT-1,-2, and 5 μg of bovine serum albumin in 20 μl of gel shift buffer (20 mM Tris-HCl [pH 7.4], 50 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 25% [vol/vol] glycerol) for 30 min on ice. The ΔCATT-1,-2 competitor was included to reduce nonspecific binding and to identify only those proteins interacting with sequences shown to be essential for promoter activity (Fig. 2). After incubation, the samples were loaded onto a 6% polyacrylamide gel prepared with 0.4× Tris-borate-EDTA (TBE) and run at a constant current of 12 mA in 0.4× TBE. Gels were then wrapped in

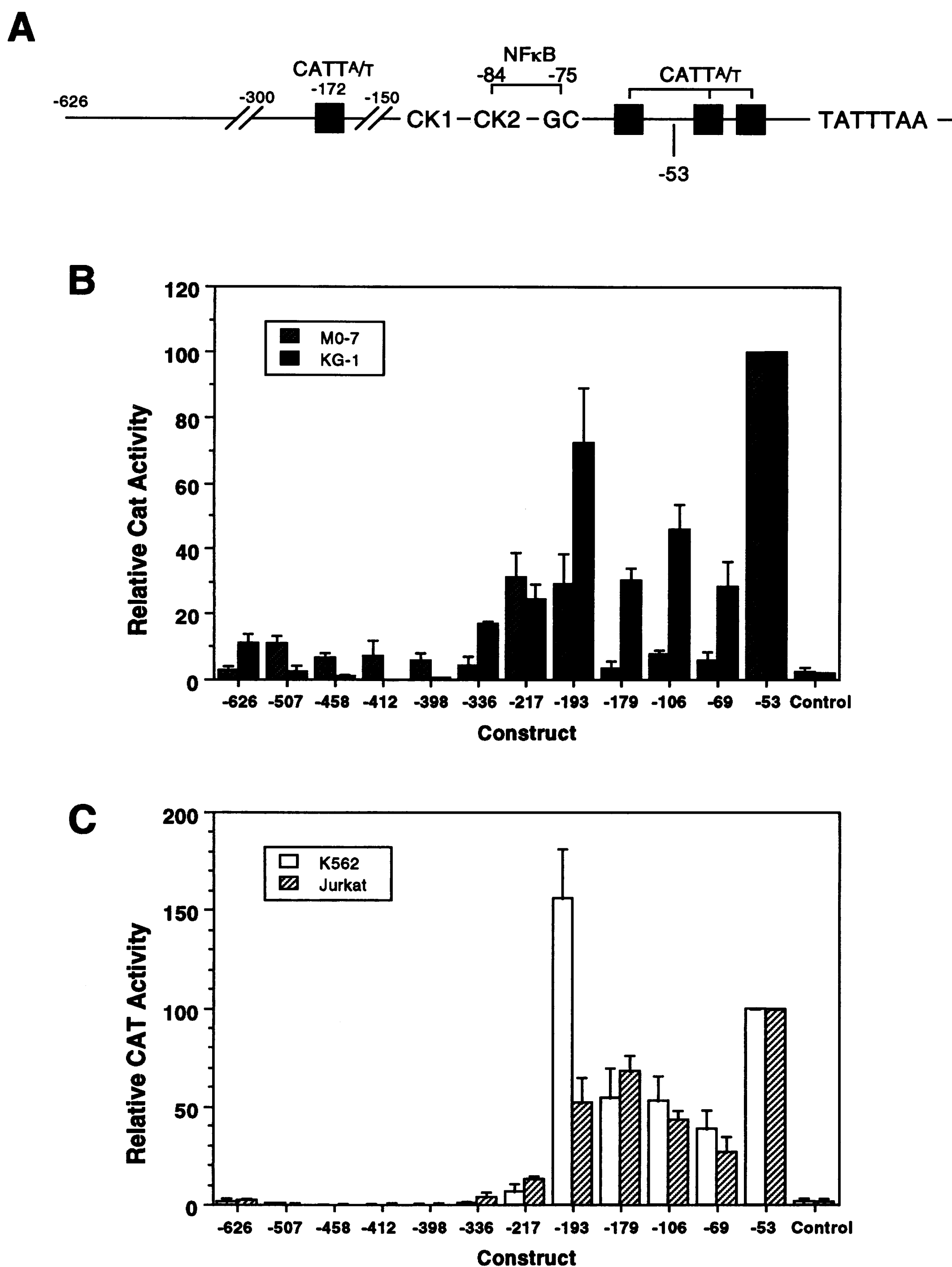

FIG. 1. Effects of nested 5′ deletion mutants on GM-CSF promoter activity. (A) Map of GM-CSF promoter showing putative regulatory elements. The full-length −626CAT construct and the various deletion mutant GM-CSF promoter CAT constructs are diagrammed. The locations of sequence motifs held in common with the promoters of related lymphokine genes are highlighted. CATT represents the sequence CATT$^{A}_{T}$ present in four copies in the human GM-CSF 5′-flanking region. CK1 and CK2 (for conserved cytokine sequences 1 and 2) are from Miyatake et al. (16). GC represents a guanine-cytosine-rich stretch of sequence. NFκB represents a potential binding site for the factor NF-κB. This site is present on the noncoding strand between nt −75 and −84. For reference, the position of the TATA box is also included. (B) Activity of 5′ deletion mutant promoter constructs in AML cells. The absolute CAT activity for the −53CAT construct was 10 to 25% acetylation of chloramphenicol for MO-7 and 14 to 35% for KG-1. Data for each experiment were normalized to that of construct −53CAT and are presented as the means ± standard errors of the mean of at least three experiments. (C) Activity of 5′ deletion mutant promoter constructs in K562 and Jurkat cells. The absolute CAT activity for the −53CAT construct was 4 to 16% for K562 and 17 to 42% for Jurkat. Data for each experiment were normalized to that of construct −53CAT and are presented as the means ± standard errors of the mean of at least three experiments.

Construct	Sequence	KG-1	MO-7	JURKAT	K562
		RELATIVE CAT ACTIVITY			
-626CAT	G-C-A-T-T-T-T-G-T-G-G-T-C-A-C-C-A-T-T-A-A-T-C-A-T-T-T-C-C-T-C-T (CATT-3, CATT-2, CATT-1)	7.6±1.1	6.4±2.1	0.1±0.6	0.1±0.6
-626CATΔCATT-1,-2	G-C-A-T-T-T-T-G-T-G-G-T-C-A-C-a-A-g-g-g-A-T-a-g-a-a-g-C-t-T-C-T	0.3±0.1	1.9±1.4	—	—
-626CATΔCATT-2	G-C-A-T-T-T-T-G-T-G-G-T-C-A-C-g-A-a-T-A-A-T-C-A-T-T-T-C-C-T-C-T	0.5±0.1	2.1±1.9	—	—
-626CATΔCATT-1	G-C-A-T-T-T-T-G-T-G-G-T-C-A-C-C-A-T-T-A-A-c-g-A-a-T-T-C-C-T-C-T	0.5±0.2	1.4±1.2	—	—
-53CAT	G-T-C-A-C-C-A-T-T-A-A-T-C-A-T-T-T-C-C-T-C-T	100	100	100	100
-53CATΔCATT-1	G-T-C-A-C-C-A-T-T-A-A-c-g-A-a-T-T-C-C-T-C-T	12.2±3.8	10.2±3.2	35.7±3.1	14.7±8.8

FIG. 2. Activity of site-directed substitution and 5′ deletion mutant promoter constructs. The shaded areas and lowercase characters represent regions of base pair substitution. Data are presented as the means ± standard errors of CAT activity (percent acetylation) of at least three experiments. The absolute CAT activity for the −53CAT construct was 15 to 35% acetylation of chloramphenicol for KG-1, 10 to 15% for MO-7, 23 to 40% for Jurkat, and 9 to 17% for K562 cells.

Saran Wrap and irradiated for 5 to 10 min with a Hoefer Mighty Bright UV transilluminator (300 nm). Shifted bands were visualized by exposure on Kodak X-Omat film for 3 to 18 h, excised, and eluted from the polyacrylamide by mixing for 6 to 18 h in sodium dodecyl sulfate (SDS) sample loading buffer (125 mM Tris-HCl [pH 6.8], 6.1% [wt/vol] SDS, 20% [vol/vol] glycerol). Cross-linked species were resolved on an SDS–10% polyacrylamide gel alongside ^{14}C-labeled protein size standards. Gels were fixed in 30% methanol–10% acetic acid, dried, and exposed on Kodak X-Omat film.

RESULTS

Analysis of 5′ deletion mutants in unstimulated cells. Analysis of the activity of a family of 5′ nested deletion mutants in the AML cell lines KG-1 and MO-7 is shown in Fig. 1B. The highest promoter activity was generated by the smallest construct (−53CAT), demonstrating the presence of a strong constitutive positive regulatory element within 53 bp of the start site of transcription. This element exhibited 45- to 60-fold-greater activity than the negative control plasmid. Interestingly, the next largest construct (−69CAT), which contains an additional 16 bp of promoter sequence, showed considerably reduced activity (17-fold in MO-7 cells), suggesting the presence of a repressive element within this region. A second positive regulatory region is seen between nt −179 and −193, and an additional negative regulatory activity is seen between nt −193 and −336.

When the same constructs were electroporated into non-AML cells (the CML cell line K562 and the T-cell line Jurkat) a similar pattern is seen (Fig. 1C). Sequences within 53 bp of the start site of transcription contain a very strong positive element in all cell lines which is repressed by addition of sequences to nucleotide −69. Sequences between −179 and −193 also exhibit a positive activity in K562 cells but not in Jurkat cells, suggesting that this activation may be myeloid cell specific. A second inhibitory element is also seen between nt −193 and −217.

Analysis of the downstream positive regulatory element. Given the strength of the positive and negative regulatory elements present within −69 bp of the start site of transcription and their proximity to each other, we focused our attention on these sequences. We have previously demonstrated that a repeat of the sequence CATT$^{A}_{T}$ downstream of bp 53 (−48 to −37) is essential for promoter activity in T cells (19, 20) and fibroblasts (21), so we examined whether these sequences also mediate positive activity in AML cells.

The results, shown in Fig. 2, demonstrate that mutation of both CATT$^{A}_{T}$ elements (construct −626CATΔCATT-1,-2) or either element alone (constructs −626CATΔCATT-2 and −626CATΔCATT-1) resulted in loss of the low-level constitutive promoter activity seen in the context of 626 bp of promoter sequence. This was confirmed by examination of the effect of a 3-bp substitution mutation in the downstream CATT$^{A}_{T}$ motif in the context of the −53CAT plasmid (construct −53CATΔCATT-1) in KG-1, MO-7, Jurkat, and K562 cells. This mutation reduced constitutive promoter activity relative to that of wild-type −53CAT 7- to 10-fold in KG-1, MO-7, and K562 cells and 3-fold in Jurkat cells. These data suggest that the constitutive activity of construct −53CAT is mediated by the same sequences that mediate promoter activity in T cells and fibroblasts.

Analysis of the negative regulatory element located between bp −69 and −53. The data presented in Fig. 1 demonstrate that sequences between −69 and −53 repress the activity of this CATT$^{A}_{T}$ repeat element in all cell types. To determine the sequences mediating this negative regulatory activity, we performed additional mutagenesis of the promoter.

Results obtained with these constructs (Fig. 3) show that increasing spacing by 5 bp (construct −626CATΔBKL) significantly increased promoter activity in all cells tested. This effect was most evident in MO-7 cells, in which it mediated a 20-fold

Construct	Sequence	MO-7	KG-1	JURKAT	K562
		RELATIVE CAT ACTIVITY			
-626CAT	-626 ← T-C-C-C-T-G-G-C-A-T-T-T-T-G-T-G-G-T-C-A-C-C-A-T-T-A-A-T-C-A-T-T-T-C-C-T → (CATT-3, CATT-2, CATT-1)	6.0±3.5	4.0±1.4	0±0.3	1.6±0.9
-626CATΔBKL	-626 ← T-C-C-C-T-G-G-C-A-T-T-T-T-G-T-G(g-t-c-a-c)G-T-C-A-C-C-A-T-T-A-A-T-C-A-T-T-T-C-C-T →	125.9±17.6	21.3±3.8	7.2±2.1	18.1±9.8
-626CATΔ-53/-49	-626 ← T-C-C-C-T-G-G-C-A-T-T-T-T-G-T-G-C-A-T-T-A-A-T-C-A-T-T-T-C-C-T →	2.3±0.2	3.8±0.1	2.2±1.1	2.7±3.2
-69CAT	-69 T-C-C-C-T-G-G-C-A-T-T-T-T-G-T-G-G-T-C-A-C-C-A-T-T-A-A-T-C-A-T-T-T-C-C-T →	5.9±2.9	30.0±3.8	19.7±11.5	43.8±18.1
-69CATΔ66	-69 T-C(g-a-g)G-G-C-A-T-T-T-T-G-T-G-G-T-C-A-C-C-A-T-T-A-A-T-C-A-T-T-T-C-C-T →	ND	84.8±13.9	ND	ND
-69CATΔ60	-69 T-C-C-C-T-G-G(a-t-c-c)T-T-G-T-G-G-T-C-A-C-C-A-T-T-A-A-T-C-A-T-T-T-C-C-T →	94.1±13.9	103.6±12.2	4.8±0.8	5.3±1.4
-69CATΔ55	-69 T-C-C-C-T-G-G-C-A-T-T-T-T(c-g-t)G-T-C-A-C-C-A-T-T-A-A-T-C-A-T-T-T-C-C-T →	ND	17.2±5.1	ND	ND
-53CAT	-53 G-T-C-A-C-C-A-T-T-A-A-T-C-A-T-T-T-C-C-T →	100	100	100	100

FIG. 3. Effects of 5′ deletion and site-directed substitution mutant promoter constructs on negative regulatory activity in leukemia cell lines. For clarity, the 5 bp that were duplicated (construct −626CATΔBKL) are in lowercase characters. The shaded area represents the regions of base pair substitution. Underlining is used to indicate the CATT$^{A}_{T}$ repeats. CAT activity is represented as the percentage of that observed with the maximally active construct, −53CAT, after subtraction of background determined by an inactive promoter. Data are presented as the means of at least four experiments ± the standard errors of the mean. The absolute CAT activity for the −53CAT construct was 15 to 35% acetylation of chloramphenicol for KG-1, 10 to 15% for MO-7, 23 to 40% for Jurkat, and 9 to 17% for K562 cells.

increase in promoter activity. These data suggest that this insertion disrupts an existing negative regulatory element. However, it also creates a repeat of the sequence GTCAC and may alter the spacing between positive and negative regulatory elements. Deletion of 5 bp from this region (construct −626CATΔ-53/-49) resulted in slightly reduced promoter activity in KG-1 and MO-7 cells (Fig. 3), indicating that neither the spacing nor these 5 bp are essential for the activity of the negative element. This mutation had very little effect in K562 and Jurkat cells. The data further demonstrate that mutation of the third CATT$^{A}_{T}$ sequence (construct −69CATΔ60) considerably enhanced promoter activity relative to that of construct −69CAT in the AML cell lines KG-1 (4-fold) and MO-7 (16-fold). In the CML cell line K562 and in Jurkat T cells, this mutation reduced promoter activity. These data suggest that sequences around the CATT-3 motif form an important part of the negative regulatory element between nucleotides −53 and −69 in AML cells but that in non-AML cells this element mediates a positive activity. More detailed analysis with KG-1 cells demonstrated that mutation further upstream (−69CATΔ66) caused a similar disruption of negative activity. By contrast, mutation downstream (−69CATΔ55) had little effect on promoter activity.

Competition cotransfection. It appears that sequences from −67 to −59 (including CATT-3) contain an element that mediates a negative regulatory influence in KG-1 and MO-7 cells and that sequences containing the two downstream CATT$^{A}_{T}$ elements are required for positive activity. This sequence homology suggests the potential for cooperative or competitive interaction between these motifs. To examine this possibility, we performed competition cotransfection experiments using a plasmid which lacks the CAT gene and in which the downstream region (CATT-1 and CATT-2) had been mutated (construct −69pUCΔCATT-1,-2 [Fig. 4A]). A construct containing mutations in both the positive and the negative elements (−69pUCΔCATT-1,-2,-3) was used as a control, and plasmid −53CAT was used as the reporter.

The results (Fig. 4B) demonstrated that in KG-1 and MO-7 cells, CAT activity increased with increasing concentrations of competitor plasmid −69pUCΔCATT-1,-2. No corresponding increase in CAT activity was seen with the control plasmid. Similarly, no change in CAT activity was seen in Jurkat or K562 cells with any of the competitor plasmids (data not shown).

These data suggest that the sequences within construct −53CAT can bind both positively and negatively acting proteins and that the binding of the inhibitory protein(s) can be abolished by the upstream negative regulatory element, result-

FIG. 4. Competition cotransfection. (A) Sequences of cotransfected constructs. The shaded areas represent the regions of base pair substitution, and the CATT$^{A}_{T}$ repeats are shown in the rectangles. (B) Competition cotransfection data. Competition cotransfection data are shown as percent maximum acetylation. Each line represents data combined from two experiments. Each point represents 2.5 μg of the −53CAT reporter construct and 0, 2.5, 7.5, 12.5, 17.5, 20, or 22.5 μg of competitor −69pUCΔCATT-1,-2 or 22.5 μg of control plasmid −69pUCΔCATT-1,-2,-3. The total amount of plasmid DNA in each sample used for electroporation was adjusted to 25 μg with pUC18.

ing in increased promoter activity. This also suggests that distinct proteins are responsible for the positive and negative regulatory activities of this region.

EMSA and UV cross-linking analysis of the negative regulatory factor. The results described above predict the existence of a negative regulatory factor capable of binding both to sequences 3′ of bp −53 and to sequences between −69 and −53. To test this prediction, we prepared oligonucleotide probes containing one or other of these regions for use in EMSA analysis.

By using heparin agarose-fractionated KG-1 nuclear extract and a radiolabeled oligonucleotide probe spanning the downstream element (bp −52 to −33), two specific EMSA complexes were observed (one in the 0.3 M KCl eluate and one eluted at 0.5 M KCl [Fig. 5]). The specific band seen in the 0.3 M eluate had low mobility and was not inhibited by control oligonucleotides or by the oligonucleotide containing the −53 to −69 negative regulatory sequences. The specific band seen at 0.5 M KCl was also not inhibited by control oligonucleotides; however, unlike the 0.3 M KCl activity, this band was inhibited by the upstream element. Thus, it appears that the 0.3 M KCl complex is specific for the −52 to −33 sequences (which overlap the positive regulatory element), while the protein(s) responsible for the 0.5 M KCl complex is capable of specific binding to sequence elements contained in either the −52 to −33 or the −69 to −48 probes as predicted by the competition cotransfection. In the reciprocal experiment using the upstream sequences as a probe, no binding activity was detected in the 0.3 M KCl fraction, while a species of identical mobility was detected in the 0.5 M KCl eluate (data not shown).

We have used UV cross-linking to characterize the DNA-binding proteins present in KG-1 cells that are contained in these specific complexes. The 0.3 M KCl complex contained a single 105- to 110-kDa species (Fig. 5B, lane 1), whereas the 0.5 M KCl complex contained a species of 53 kDa (Fig. 5B, lane 2). An identical species was detected in cross-linking of the 0.5 M KCl complex with the upstream element as a probe (Fig. 5b, lane 3). Allowing for single-stranded DNA probe size (approximately 8 kDa), the proteins responsible for these cross-linked complexes are 97 to 102 kDa for 0.3 M KCl and 45 kDa for 0.5 M KCl.

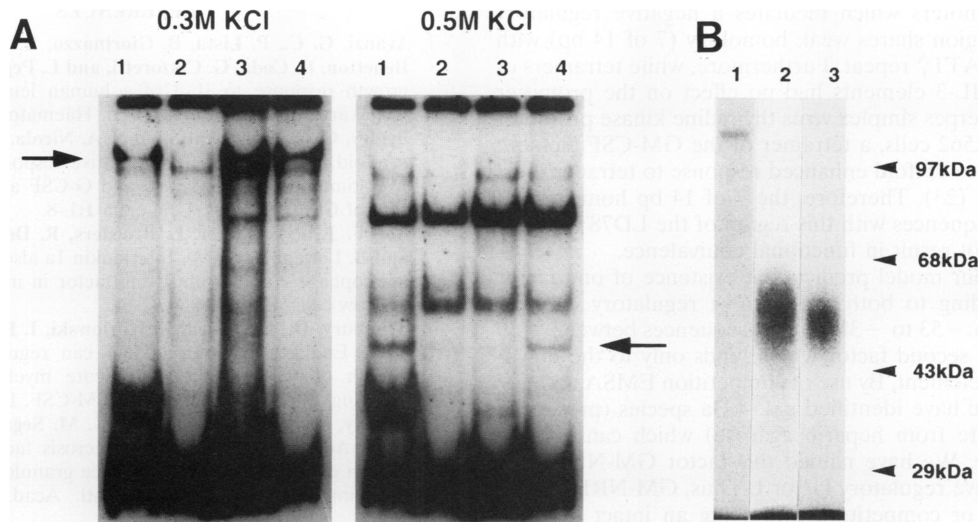

FIG. 5. Gel EMSA and UV cross-linking. (A) Gel EMSA with −52 to −33 probe and heparin agarose chromatography fractions. All lanes include 1 ng of radiolabeled −52 to −33 oligonucleotide probe, 500 ng of unlabeled ΔCATT-1-2 oligonucleotide, and 2 μg of fractionated KG-1 nuclear extract. Lane 1, 500 ng of unlabeled −69Δ-1-2-3 (control) oligonucleotide; lane 2, 500 ng of unlabeled −52 to −33 oligonucleotide (competition with self); lane 3, 500 ng of unlabeled −69 to −48 oligonucleotide (competition with the upstream element); lane 4, 500 ng of unlabeled control oligonucleotide CRE2. (B) UV cross-linking of specific EMSA complexes. Lane 1, cross-linking of the band with exclusive specificity for the positive element (panel A, 0.3 M KCl, lane 1); lane 2, cross-linking of the band with specificity for both GM-CSF promoter probes (panel A, 0.5 M KCl, lane 1; generated with probe −52 to −33); lane 3, cross-linking of the band with specificity for both GM-CSF promoter probes (generated with probe −69 to −48), gel shift not shown.

DISCUSSION

A number of studies have detected expression of GM-CSF mRNA and/or protein in cultured AML cells (36, 37). It has also been demonstrated that fresh primary human AML cells express IL-1 mRNA and that endogenous (or exogenous) IL-1 may induce GM-CSF expression in certain normal and leukemic cells (3, 4). The majority of AML blast cells express a high-affinity receptor for GM-CSF (9, 13) and demonstrate a proliferative response to GM-CSF in vitro (2). Thus, production of GM-CSF by AML cells may establish an autocrine loop, allowing these cells to escape normal growth-regulatory mechanisms. It has also been proposed that GM-CSF expression by leukemic cells is a reflection of its expression by normal immature myeloid cells, as part of a mechanism to prolong and enhance the response of progenitor cells to primary differentiation stimuli (25). Both models suggest that the mechanisms controlling GM-CSF expression in AML cells differ from those of other cells which are not responsive to the cytokine.

Our studies have focused on two regulatory elements present within 69 bp of the start site of transcription. The data reveal that while the positive regulatory elements in different cell types appear to be similar, the mechanisms which repress this activity in AML cell lines are distinct from those seen in T-lymphocyte and CML cell lines. Our results demonstrate the presence of a strong constitutive promoter element present within 53 bp of the GM-CSF cap site which is active in all cell types. The critical sequences mediating this activity are centered around the CATT$^{A}_{T}$ repeats from nt −48 to −37. We previously identified this region as containing full inducible GM-CSF promoter activity in phytohemagglutinin-stimulated T cells (20, 22) as well as constitutive and tetradecanoyl phorbol acetate-stimulated GM-CSF promoter activity in fibroblasts (21). These data have been confirmed by Miyatake et al. (17) with the murine GM-CSF promoter CATT$^{A}_{T}$ repeat motif. These sequences are completely conserved in the murine GM-CSF promoter (28) and exhibit very high homology (13 of 15 bp) with sequences in the 5′-flanking region of the human and murine IL-5 genes (6).

We have also demonstrated the presence of several negative regulatory elements, one of which is located between bp −67 and −59 immediately upstream of the positive regulatory element. Examination of this negative regulatory element by mutational and competition cotransfection analysis suggests a model in which a critical positive factor(s) is prevented from binding to the positive regulatory element by binding of a negative factor to overlapping sequences. The internal deletion/insertion mutants shown in Fig. 3 indicate that increasing the spacing between the two negative sites abolishes their ability to repress promoter activity. Decreasing the spacing by deletion of 5 bp had little effect on promoter activity with perhaps a slight increase in repression being seen in the AML cells. Cotransfection studies with KG-1 and M-O7 cells suggest that the upstream negative element present on the pUC-based plasmid (−69pUCΔCATT-1,-2) competes for binding of the inhibitory factor to the downstream element present in the reporter plasmid (−53CAT), thereby increasing promoter activity. In recent studies, we have seen similar competition by the −69ΔCATT-1-2 plasmid in a third AML cell line, TF-1 (data not shown). This mechanism of repression appears to be cell type restricted in that competition experiments using −69pUCΔCATT-1,-2 (which contains mutations in the positive element and the downstream negative element) in Jurkat and K562 cells showed no effect on reporter gene expression of construct −53CAT (data not shown). In addition, reporter gene construct −69CATΔ60 (which contains mutations in the CATTT sequence located in the upstream element) showed reduced rather than increased activity in the CML cell line K562 and the T-lymphoblast Jurkat cells.

Our model is distinct from that recently proposed by Nomiyama et al. (23), who defined a region of the murine IL-3

and LD78 promoters which mediates a negative regulatory activity. This region shares weak homology (7 of 14 bp) with the GM-CSF CATT$^{A}_{T}$ repeat. Furthermore, while tetramers of the LD78 and IL-3 elements had no effect on the promoter activity of the herpes simplex virus thymidine kinase promoter in Jurkat and K562 cells, a tetramer of the GM-CSF element conferred a 14- to 15-fold enhanced response to tetradecanoyl phorbol acetate (23). Therefore, the 7 of 14 bp homology of the GM-CSF sequences with this region of the LD78 negative element does not result in functional equivalence.

In contrast, our model predicts the existence of one factor capable of binding to both the positive regulatory element located between −53 to −39 and the sequences between −59 and −67 and a second factor which binds only to the downstream positive element. By use of competition EMSA and UV cross-linking, we have identified a 45-kDa species (present in the 0.5 M eluate from heparin agarose) which can bind to either sequence. We have named this factor GM-NRF-I for GM-CSF negative regulatory factor I. Thus, GM-NRF-I binds only to probes or competitors containing an intact negative element. It does not bind to the ΔCATT-1-2 mutant present at a 500-fold excess in all binding reaction mixtures or to the unrelated sequences present in the CRE2 oligonucleotide. We have also detected a factor (GM-PRF-A [for GM-CSF positive regulatory factor A]; molecular mass, 97 to 102 kDa) which does not bind to sequences between −53 and −69 or to the ΔCATT-1-2 mutant and therefore appears to be specific for the positive regulatory element. We have detected a similar species in related studies of GM-CSF expression in activated T cells (unpublished data). However, in these cells four additional proteins that were not seen in AML cells were observed.

Thus, while positive transcriptional activity of the GM-CSF promoter appears to be mediated through distinct factors which bind to similar constitutively active elements, we have identified distinct cell-type-restricted mechanisms which repress this activity in AML cells. The data we have generated (Fig. 1) clearly indicate that other elements are also involved in cell-type-specific regulation of the GM-CSF promoter. Thus, sequences between −179 and −193 mediate an additional positive regulatory activity in the three myeloid cells (KG-1, MO-7, and K562) but not in the T-cell line, Jurkat.

Our data suggest a model in which transcriptionally productive binding of GM-PRF-A to the CATT$^{A}_{T}$ repeat is prevented by high-affinity binding of GM-NRF-I to sequences overlapping the critical positive element and to sequences between −59 and −67. Furthermore, the observation that a 5-bp insertion between these regulatory regions disrupts their inhibitory activity suggests that an interaction between the molecules that bind to these elements is required for repression. We propose that these cell-type-specific and additional control mechanisms regulate the expression of GM-CSF in developing normal myeloblasts and in primary AML blast cells.

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