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PU.1 (Spi-1) and C/EBPα Regulate Expression of the Granulocyte-Macrophage Colony-Stimulating Factor Receptor α Gene

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Growth factor receptors play an important role in hematopoiesis. In order to further understand the mechanisms directing the expression of these key regulators of hematopoiesis, we initiated a study investigating the transcription factors activating the expression of the granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor α gene. Here, we demonstrate that the human GM-CSF receptor α promoter directs reporter gene activity in a tissue-specific fashion in myelomonocytic cells, which correlates with its expression pattern as analyzed by reverse transcription PCR. The GM-CSF receptor α promoter contains an important functional site between positions −53 and −41 as identified by deletion analysis of reporter constructs. We show that the myeloid and B cell transcription factor PU.1 binds specifically to this site. Furthermore, we demonstrate that a CCAAT site located upstream of the PU.1 site between positions −70 and −54 is involved in positive-negative regulation of the GM-CSF receptor α promoter activity. C/EBPα is the major CCAAT/enhancer-binding protein (C/EBP) form binding to this site in nuclear extracts of U937 cells. Point mutations of either the PU.1 site or the C/EBP site that abolish the binding of the respective factors result in a significant decrease of GM-CSF receptor α promoter activity in myelomonocytic cells only. Furthermore, we demonstrate that in myeloid and B cell extracts, PU.1 forms a novel, specific, more slowly migrating complex (PU-SF) when binding the GM-CSF receptor α promoter PU.1 site. This is the first demonstration of a specific interaction with PU.1 on a myeloid PU.1 binding site. The novel complex is distinct from that described previously as binding to B cell enhancer sites and can be formed by addition of PU.1 to extracts from certain nonmyeloid cell types which do not express PU.1, including T cells and epithelial cells, but not from erythroid cells. Furthermore, we demonstrate that the PU-SF complex binds to PU.1 sites found on a number of myeloid promoters, and its formation requires an intact PU.1 site adjacent to a single-stranded region. Expression of PU.1 in nonmyeloid cells can activate the GM-CSF receptor α promoter. Deletion of the amino-terminal region of PU.1 results in a failure to form the PU-SF complex and in a concomitant loss of transactivation, suggesting that formation of the PU-SF complex is of functional importance for the activity of the GM-CSF receptor α promoter. Finally, we demonstrate that C/EBPα can also activate the GM-CSF receptor α promoter in nonmyeloid cells. These results suggest that PU.1 and C/EBPα direct the cell-type-specific expression of GM-CSF receptor α, further establish the role of PU.1 as a key regulator of hematopoiesis, and point to C/EBPα as an additional important factor in this process.

Hematopoietic growth factors and their receptors play an important role in the proliferation, differentiation, and survival of hematopoietic cells. Among these factors, functional and binding analyses suggest that the receptor for granulocyte-macrophage colony-stimulating factor (GM-CSF) is expressed specifically on myeloid cells and their precursors (8, 42, 43, 68). In myeloid cells, the GM-CSF receptor is expressed on early myeloid cells as well as on mature phagocytic cells, reflecting the ability of GM-CSF to modulate the development and activity of myeloid cells throughout differentiation (21, 50, 68). The heterodimeric GM-CSF receptor consists of at least two subunits, an α chain, which binds GM-CSF with low affinity when expressed alone, and a nonbinding β chain, which is required for the formation of high-affinity receptors and signal transduction and is shared by two other cytokine receptors, the interleukin-3 (IL-3) and IL-5 receptors (32). Functional GM-CSF receptors have been identified on primary and cultured myeloblasts from patients with acute leukemia and on neutrophils and basophils from patients with juvenile chronic myeloid leukemia (12, 43). An increased responsiveness to GM-CSF has been implicated in the pathogenesis of juvenile chronic myelomonocytic leukemia (12). However, the differential expression of the α and β subunits, their tissue specificity, and their developmental regulation on both hematopoietic and nonhematopoietic cells remain poorly understood.

Recently, the gene for the α subunit of the human GM-CSF receptor has been cloned, and its genomic structure, including the putative promoter region, has been described (35). The major transcription initiation site in hematopoietic cells was determined to be 195 bp upstream of the translation initiation site (35). As with many other genes which are regulated during myeloid cell differentiation, the sequence of the 5′-flanking region does not contain an obvious TATA motif (35). Interestingly, several purine-rich stretches, resembling PU.1 binding sites, were identified in the proximal 5′ upstream region.

PU.1 is a member of the ets family of transcription factors and is specifically expressed in myeloid and B cells (6, 24). PU.1 was first identified as the product of the Spi-1 oncogene in Friend virus-induced erythroleukemia (16, 33, 44), and overexpression of PU.1 in erythroid progenitor cells can block erythroblast differentiation (55). Recently, many target genes

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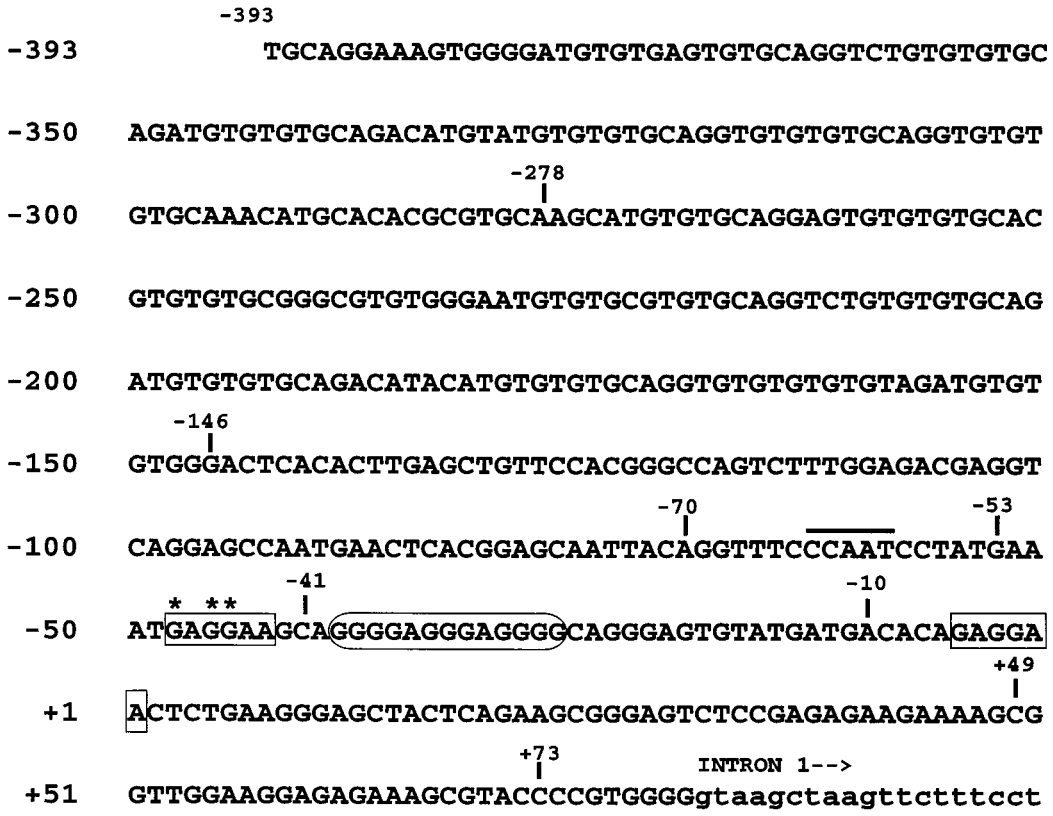


FIG. 1. Schematic representation of the GM-CSF receptor α promoter, PU.1 and C/EBPα binding sites, and deletion mutants. The DNA sequence is taken from reference 35. The position of the transcription start site is numbered +1 (35). The purine-rich region (bp −39 to −27) common to several myeloid promoters is circled (35). A GA-rich sequence (bp −5 to +1), which does not bind PU.1, and the actual PU.1 binding site identified by gel shift assay (bp −48 to −43) are boxed. The guanosine residues at bp −48, −46, and −45 which contact PU.1 as identified by methylation interference are indicated by asterisks. The CCAAT box at bp −63 to −59 is overlined. The construct -393GM-CSFr-luc contains the sequence from residue −393 to +49 cloned into the luciferase reporter plasmid pXP2. The 5′ positions of the deletion constructs are indicated by numbers above the sequence. The first intron, which starts after bp +81, is indicated by lowercase letters.

for the transcriptional regulation by PU.1 have been identified in myeloid cells (1, 10, 13, 25, 29, 34, 41, 45, 52, 62, 70). Among these is the receptor for M-CSF (70), which has a PU.1 site at nearly the identical position with respect to the transcription initiation site as a putative PU.1 site in the GM-CSF receptor α promoter. PU.1 has a crucial role for hematopoietic development, as shown by the inhibition of colony formation of human $CD34^+$ cells by PU.1 binding double-stranded oligonucleotides (66). Furthermore, gene targeting of PU.1 in mice results in a complete abrogation of fetal development of myeloid cells and lymphocytes (56).

The PU.1 protein consists of 272 amino acids, with the DNA binding domain located in the carboxyl-terminal part of the protein, while the amino terminus contains an activation domain, which has been implicated in interactions with other regulatory proteins (19, 24). In B cells, PU.1 recruits a second, B cell-specific DNA binding factor, NF-EM5, to a site important for immunoglobulin κ 3′ enhancer function (11, 46, 47). For this interaction, phosphorylation of a serine residue at position 148 is necessary (47). However, such interactions have not been shown to play a functional role in previously described myeloid targets of PU.1, such as CD11b and the M-CSF receptor (41, 70). In vitro experiments also demonstrated an interaction of the amino-terminal region of PU.1 with a conserved region in both the basal transcription factor TATA-binding protein (TBP) and the retinoblastoma (RB) protein (19), and in these TATA box-lacking, myeloid promoters, the PU.1 site could function like a TATA box to recruit the basal transcription factors to the promoters (10, 19).

Upstream of the potential PU.1 sites in the 5′-flanking region of GM-CSF receptor α are sequences representing potential binding sites for CCAAT-binding proteins. Members of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors have been shown to regulate the terminal differentiation of adipocytes and hepatocytes (5, 28). In the hematopoietic system, C/EBPα, C/EBPβ, and C/EBPδ are specifically expressed in myeloid, but not in erythroid or lymphoid cells and are differentially expressed during myelomonocytic differentiation (18, 22, 36, 57, 64). High levels of expression of C/EBPα in proliferating myeloid cells (36, 57) suggest that C/EBPα may activate genes involved in the growth and differentiation of these cells. Currently, few target genes for the regulatory function of C/EBP have been definitely identified in myeloid cells. In chicken cells, the C/EBP factor NF-M regulates the expression of the *mim-1* gene (4, 37) and of chicken myelomonocytic growth factor, a homolog of human G-CSF (18, 22, 64). The promoter of the human neutrophil elastase gene contains a functional site which resembles a C/EBP site (39). Moreover, the promoter regions of the genes for several cytokines which are expressed in macrophages, including human G-CSF, IL-6, IL-1α, IL-1β, IL-8, tumor necrosis factor alpha, and monocyte chemoattractant protein 1, contain known or predicted C/EBP binding sites (3, 36, 48).

Potential binding sites in the 5′-flanking region of an impor-

tant myeloid growth factor receptor, GM-CSF receptor α, prompted us to investigate whether PU.1 and/or C/EBP play important roles in the transcriptional activation of this key regulator of hematopoietic development. Here we demonstrate that both PU.1 and C/EBP bind to sites critical for myeloid cell-specific expression of the GM-CSF receptor α promoter. Moreover, PU.1 forms a larger complex with another protein which may be important for functional activity.

MATERIALS AND METHODS

Cell culture. Human myelomonocytic U937 cells (ATCC CRL 1593; American Type Culture Collection, Rockville, Md.), B-lymphoblastic Raji and BJAB cells, and T-lymphoblastic REX cells (provided by James Griffin, Dana-Farber Cancer Institute) and Jurkat cells were maintained in RPMI 1640 medium (Gibco, Grand Island, N.Y.) supplemented with 10% fetal calf serum (HyClone, Logan, Utah) and 2 mM L-glutamine (Gibco). Human cervical carcinoma HeLa cells (ATCC CCL 2) and monkey kidney Cos7 cells were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum (Gibco) and 2 mM L-glutamine (Gibco). The human hepatoma cell line HepG2 (provided by J. Papaconstantinou) was maintained in a 1:1 mixture of F12 medium (GIBCO) and Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 10 μg of bovine insulin per ml. CV-1 cells, an African green monkey kidney cell line (ATCC CCL 70), were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (Gibco) and 2 mM L-glutamine.

RT-PCR analysis of GM-CSF receptor α gene expression. Total RNA was isolated from cell lines by the guanidium isothiocyanate-CsCl method (53). First-strand cDNA was synthesized from 30 ng of total RNA, using 200 U of Moloney murine leukemia virus reverse transcriptase in a 20-μl reaction mixture containing 1 mM deoxynucleoside triphosphates (dNTPs), 1 U of RNasin per μl, 100 pmol of random hexamers (Pharmacia), and 10 mM dithiothreitol in 1× buffer (50 mM Tris HCl [pH 8.3], 75 mM KCl, 6 mM $MgCl_2$). The reaction mixture was incubated at 23°C for 10 min, 42°C for 60 min, and 94°C for 5 min. PCR was performed in a 30-μl reaction volume containing 2 μl of the reverse transcription (RT) product, 40 pmol of each primer, 200 μM dNTPs, and 2 U of *Taq* polymerase in 1× buffer (10 mM Tris HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin). Each cycle of PCR included 1 min of denaturation at 94°C, 1 min of annealing at 60°C for the GM-CSF receptor α primers (5′-CTTCTCTCTGACCAGCA-3′ and 5′-ACATGGGTTCCTGAGTC-3′) (15) or at 50°C for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (5′-CCATGGAGAAGGCTGGGG-3′ and 5′CAAAGTTGTCATGGATGACC-3′) (2), and 90 s of extension at 72°C. A control for DNA contamination, containing water instead of cDNA, was used in each RT-PCR.

To ensure that the RT-PCR signal was linear with respect to the RNA input, we performed a kinetic analysis by varying the number of cycles of amplification for each primer set and the amount of RNA input (66). On the basis of this analysis, we used 20 cycles for the GAPDH primers and 27 cycles for the GM-CSF receptor primers. RT-PCR products were separated on a 1.5% agarose gel and blotted on a Biotrans+ membrane (ICN) in 0.4 M NaOH. The blots were dried, prehybridized, and hybridized in 0.5 M $NaPO_4$ (pH 7.2)–7% sodium dodecyl sulfate (SDS)–1% bovine serum albumin at 65°C with GM-CSF receptor α cDNA and GAPDH cDNA as probes. The membranes were washed in 2× SSC (2× SSC is 0.15 NaCl plus 0.015 M sodium citrate)–0.1% SDS at room temperature for 5 min, and in 0.2× SSC–0.1% SDS at 65°C for 10 min. Exposures were performed in the linear range at −80°C with an intensifying screen.

Isolation of the human GM-CSF receptor α promoter. The sequence 5′ of exon 1 of the human GM-CSF receptor α gene was amplified and cloned from *Bgl*II-digested human genomic DNA (peripheral blood of a normal donor) by PCR based on the published sequence (35) with primer A (5′-GTGGGATCCTGCAGGAAAGTGGGGAT-3′) and primer B (5′-GTGGATCCGGTACGCTTTCTCTCCT-3′). The synthesized fragment was 466 bp long, extending from bp −393 to +73 with the transcription start site designated +1 (Fig. 1) (35). A second PCR fragment from bp − 393 to +49 was generated by using primer A and primer C (5-′GTGGTACCGCTTTTCTTCTCTCGGA-3′) and the 466-bp-containing plasmid as a template. This PCR fragment was digested with *Bam*HI and *Kpn*I and cloned into the promoterless luciferase vector pXP2 (38). The sequence of this fragment matched the published genomic sequence. This construct is referred to as -393GM-CSFr-luc.

Transient transfections. U937, Raji, and Jurkat cells were transfected by electroporation in IMDM medium (GIBCO) at 960 μF and 300 V, and Rex cells were transfected at 280 V, with a Bio-Rad Genepulser. The cells were harvested 7 h posttransfection into 0.25 ml of lysis buffer, and 0.1 ml of this lysate was added to 0.3 ml of assay buffer for luciferase assays. The details of the transfection and luciferase assay procedures were as described previously (40). The transfection efficiency was normalized to the levels of growth hormone produced by 2 μg of cotransfected plasmid containing the cytomegalovirus promoter directing human growth hormone gene expression (CMV-hGH). Growth hormone concentrations were measured by radioimmunoassay (Nichol's Institute, San Juan Capistrano, Calif.). Data are expressed as the fold induction over that with the plasmid pXP2.

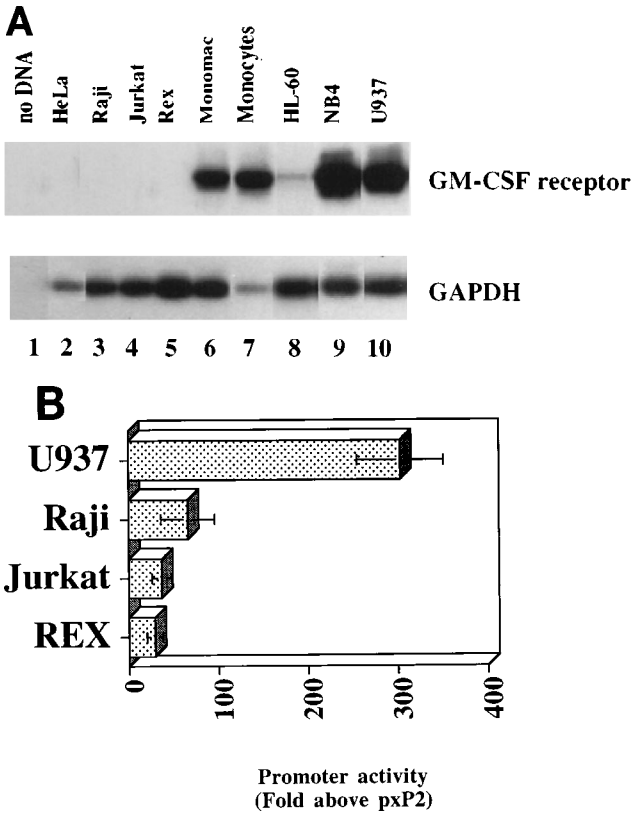


FIG. 2. (A) Expression of GM-CSF receptor α. RNA from the following sources was reverse transcribed, amplified by PCR with primers for GM-CSF receptor α or GAPDH, and hybridized with the respective cDNA probe as described in Materials and Methods: lane 1, no-RT control; lane 2, HeLa cells; lane 3, Raji B cells; lane 4, Jurkat T cells; lane 5, Rex T cells; lane 6, Monomac (monocytic) cells; lane 7, monocytes isolated from human peripheral blood; lane 8, HL-60 (promyelocytic) cells; lane 8, NB4 (promyelocytic) cells; lane 9, U937 (myelomonocytic) cells. (B) Tissue specificity of the GM-CSF receptor α promoter in transient transfections. -393GM-CSFr-luc, as well as the promoterless vector pXP2, was transfected into the GM-CSF receptor α-expressing cell line U937 and into the GM-CSF receptor-negative cell lines Raji, Jurkat, and Rex. The transfection efficiency was normalized by cotransfecting CMV-hGH. Data are shown as fold induction over the level with plasmid pXP2 and represent the means of three to six independent experiments. The standard errors of the means are indicated by the error bars. Results similar to those for Raji B cells were also obtained with the BJAB cell line, but they are not presented because the experiment was performed only once.

Nuclear extracts. Nuclear extracts from U937, Raji, Jurkat, and Rex cells were prepared by the method of Dignam et al. (7), modified as described previously (41), with the addition of 1 mM diisopropylfluorophosphate (Sigma) to buffers A and C. Extracts of Cos7 and HepG2 cells were prepared by the following method. Adherent cells (10^6 to 10^7 cells per 100-mm-diameter tissue culture plate) were washed twice with phosphate-buffered saline (PBS) (Sigma), scraped from the plate, pelleted by centrifugation at 200 × *g*, resuspended in 1 ml of PBS, and transferred to microcentrifuge tubes. The cells were pelleted again and resuspended in 400 μl of cold hypotonic buffer A (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride), and allowed to swell for 15 min on ice. The cells were lysed by being vortexed for 10 s, and the nuclei were pelleted by centrifugation at 12,000 × *g* for 10 s in a microcentrifuge. The supernatant was removed, and the nuclei were resuspended in 40 μl of cold buffer C (20 mM HEPES [pH 7.9], 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) and incubated on ice for 20 min, with flicking of the tube several times. The nuclei were pelleted at 4°C and 12,000 × *g* for 3 min, and the supernatant was removed and stored at −80°C. Analysis by the Bradford assay (Bio-Rad) showed that the extracts contained 2 to 3 mg of protein per ml.

EMSA. ^{32}P-labeled double-stranded oligonucleotides for electrophoretic mo-

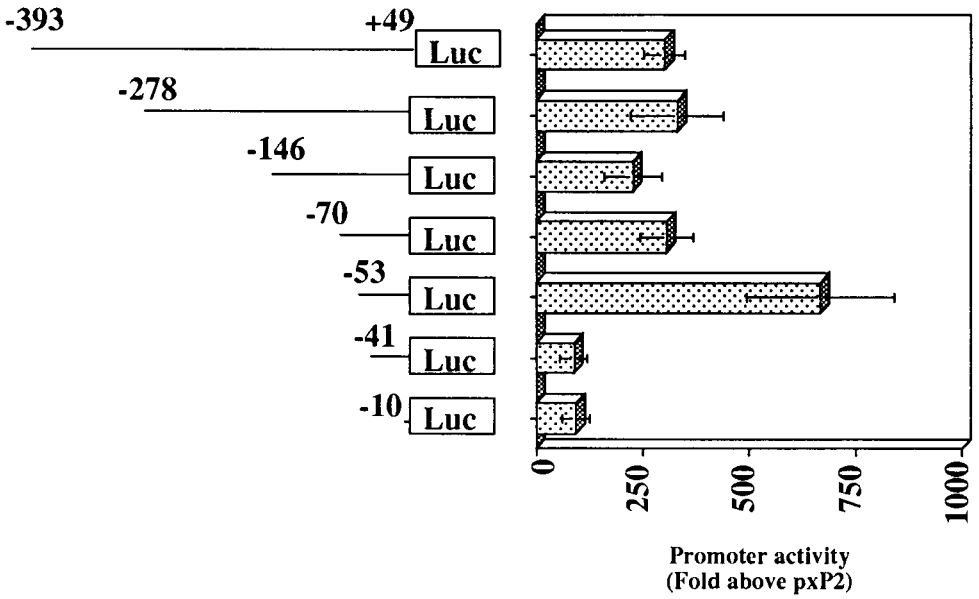


FIG. 3. Identification of critical *cis* elements in the GM-CSF receptor α promoter. A series of 5′ deletion constructs was generated and transiently transfected into U937 cells. Four to eight independent experiments were performed, and the average fold luciferase activity over the level with pXP2 and standard errors were calculated. Luciferase activity was normalized to growth hormone produced by a cotransfected CMV-hGH plasmid.

bility shift assay (EMSA) were prepared as previously described (41), and 0.5 ng (specific activity, 5×10^8 cpm/μg) was used per reaction. Nuclear extracts (10 μg) were preincubated at 4°C for 30 min in a volume of 20 μl with 2 μg of poly(dI-dC) in 10 mM HEPES (pH 7.9)–50 mM KCl–5 mM $MgCl_2$–1 mM dithiothreitol–1 mM EDTA–5% glycerol. As described in the figure legends, unlabeled competitor oligonucleotides (50 ng = 100-fold excess) were included in this 30-min preincubation. For supershift experiments, 1 μl of either specific polyclonal antiserum or normal rabbit serum was added to the preincubation mixture. A rabbit polyclonal antibody recognizing the amino terminus of PU.1 (amino acids 33 to 45 of the murine protein) was provided by Richard Maki (24, 46), and an antiserum raised against the full-length PU.1 protein was a gift from David Kabat. Rabbit antisera raised against the carboxyl four-fifths of C/EBPα, against the carboxyl-terminal 18 amino acids of C/EBPβ, and against full-length C/EBPδ were provided by Steven McKnight. An Oct-1 peptide antiserum (Santa Cruz Biotechnology) served as a control. Antiserum raised against the full-length RB protein (N9 serum) and preimmune serum were provided by Bill Kaelin. In vitro transcription and translation of PU.1, PU.1 deletion mutants, and other ets family members were performed with a rabbit reticulocyte lysate system (TnT system; Promega) as previously described (66), and 1 μl was added in the 20 μl reaction mixture instead of adding the nuclear extract. Reaction mixtures were then electrophoresed at 10 V/cm on a 5.2% polyacrylamide gel in 0.5× TBE (45 mM Tris-borate, 1 mM EDTA) at 4°C.

Methylation interference analysis. Twenty-five picomoles of an oligonucleotide spanning bp −70 to −54 (Fig. 1) was end labeled with 75 μCi of [γ^{32}-P]ATP by T4 kinase (10 U) (New England Biolabs) in a total volume of 20 μl for 60 min at 37°C. After heat inactivation at 65°C for 20 min, PCR was performed by adding 25 pmol of primer C, 2 ng of -393GM-CSFr-luc as the template, and KCl and dNTPs to final concentrations of 50 mM and 50 pM, respectively, in a total volume of 100 μl. The amplification procedure included denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min during 35 PCR cycles. The PCR product was then isolated after electrophoresis in a 1% agarose gel. The PCR fragment was partially methylated by treatment with dimethyl sulfate as previously described (30, 63, 70), subjected to a binding reaction which was scaled up fivefold from the EMSA conditions described above, and incubated on ice for 20 min. The samples were then electrophoresed on low-ionic-strength 4% polyacrylamide gels. The complexes in the free and bound fractions were visualized by autoradiography, excised from the gel, and isolated by electroelution. Each sample was extracted twice with phenol-chloroform and once with chloroform and ethanol precipitated. The pellet was resuspended in 150 μl of 1 M piperidine and incubated at 90°C for 30 min. Piperidine was removed by butanol precipitation and drying in a speed vacuum apparatus. Samples with equivalent amounts of radioactivity were subjected to electrophoresis on an 8% polyacrylamide-urea sequencing gel and visualized by autoradiography.

Construction of plasmids with deletions, with site-directed mutations, and with the PU.1 binding site upstream of the thymidine kinase (TK) promoter. Deletion constructs of the GM-CSF receptor α promoter were generated by PCR with -393GM-CSFr-luc as the template with primer C (described above) and the respective 5′-3′ primers containing a 5′ *Bam*HI site as indicated in Fig. 1. The PCR product was digested with *Bam*HI and *Kpn*I and ligated into *Bam*HI-*Kpn*I-digested luciferase vector pXP2 (38). Mutated GM-CSF receptor α promoter fragments were also generated by PCR (26). For generation of -53GM-CSFr(GM-1)-luc, containing mutations in the PU.1 site at positions −48, −46, and −45, the plasmid -393GM-CSFr-luc served as the template and primer C and 5′-GTGGATCCGAAATcAccACGCAGGG-3′ were used as primers (mutated bases are shown in lowercase). For construction of -70GM-CSFr(GM-1)-luc, the resulting PCR fragment then served as a primer for the second PCR, using this fragment and 5′-AGGTTTCCCAATCCTAT-3′ as primers and -393GM-CSFr-luc as the template. The plasmid -70GM-CSFr(GM-2)-luc, containing mutations in the CCAAT site at positions −60 and −61, was generated by PCR with primer C and 5′-AGGTTTCCCggTCCTAT-3′ with -393GM-CSFr-luc as the template. To generate the double PU.1–C/EBP mutant -70GM-CSFr(GM-1/2), -70GM-CSFr(GM-1)-luc was used instead as the template. The final PCR products were digested with *Bam*HI and *Kpn*I and ligated into *Bam*HI-*Kpn*I-digested pXP2. The plasmids PU.1-T81-luc and mutPU.1-T81-luc were constructed from oligonucleotides with wild-type and GM1 mutant sequences, respectively, from positions −53 to −36, with *Xho*I and *Sal*I restriction sites (5′-tcgagGAAATGAGGAAGCAGGGGg-3′ and 5′-tcgacCCCCTGCTTCCT CATTTCc-3′, 5′-tcgagGAAATcAccAAGCAGGGGg-3′ and 5′-tcgacCCCCT GCTTggTgATTTCc-3′). These were cloned into the *Sal*I site of pBluescript KS(−) (Strategene). A single wild-type PU.1 site was subcloned into the *Bam*HI-*Sal*I site of pT81-luc, and a single PU.1 mutant GM1 sequence was subcloned into the *Hin*dIII-*Sst*I site of pT81-luc (38). The sequences of all constructs were confirmed by the dideoxy chain termination method.

Transactivation. HepG2 and CV-1 cells were transfected by the calcium phosphate coprecipitation method as previously described (14) with 10 μg of reporter plasmid; with expression vectors containing full-length PU.1 (24), amino-terminally deleted forms of the PU.1 protein (N133 PU.1 or N70 PU.1) (59), or the C/EBP expression vector MSV-C/EBPα (5); and with 0.25 μg of CMV-hGH plasmid and sonicated double-stranded salmon sperm DNA, to a total of 20 μg of DNA. Luciferase assays were performed 40 h after transfection. Titration of expression vectors over the range of 1 to 8 μg did not produce any significant difference in results.

RESULTS

GM-CSF receptor α is specifically expressed in myelomonocytic cell lines. The expression of the GM-CSF receptor α chain and its regulation during hematopoietic differentiation have not been extensively characterized. As a basis for the subsequent comparative analysis of the cell-type-specific activity of the 5′-flanking region, we determined the mRNA expression of GM-CSF receptor α in the hematopoietic system. We analyzed several myeloid and nonmyeloid cell lines. We ob-

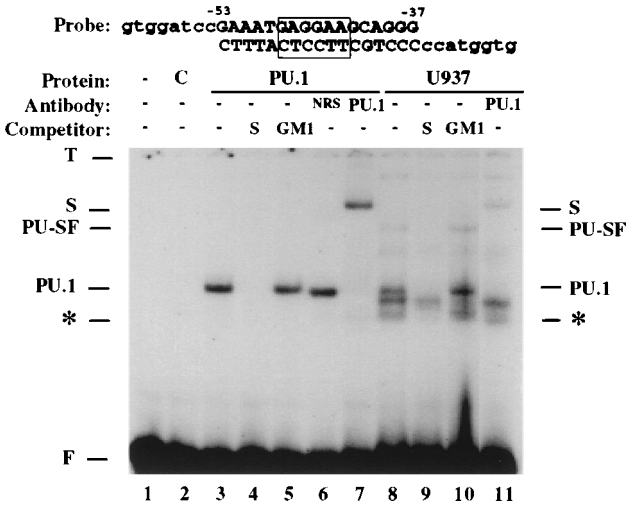


FIG. 4. Identification and characterization of PU.1 binding to the GM-CSF receptor α promoter by gel shift assay. A double-stranded GM-CSF receptor α promoter oligonucleotide extending from position −53 to −37 was end labeled with [γ-^{32}P]ATP and incubated with 2 μg of double-stranded poly(dI-dC) in the absence of nuclear protein (lane 1) or in the presence of 1 μl of unprogrammed reticulocyte lysate (lane 2), 1 μl of reticulocyte lysate containing in vitro-translated PU.1 (lanes 3 through 7), or 10 μg of nuclear protein prepared from U937 cells (lanes 8 through 11). The following unlabeled double-stranded competitor oligonucleotides were added at a 100-fold molar excess over the probe oligonucleotide: GM-CSF receptor α bp −53 to −37 (self [S]) (lanes 4 and 9) and GM-CSF receptor α mutated PU.1 site oligonucleotide (GM1) (lanes 5 and 10). In lanes 7 and 11, antiserum recognizing the amino terminus of PU.1 was added to the reaction mixture as described in Materials and Methods; in lane 6, normal rabbit serum (NRS) was added as a control. Abbreviations on the sides of the gel: T, top of the gel; S, supershifted PU.1-DNA complex with PU.1 antibody; PU.1, gel-shifted complex formed with full-length PU.1; F, unbound free oligonucleotide probe; PU-SF, complex formed by PU.1 with another protein in nuclear extracts; *, complex formed by proteolytic product of PU.1 as previously described (6, 41, 70). The sequence of the GM1 mutated PU.1 site oligonucleotide is shown in Fig. 9.

tained only a very weak signal with Northern (RNA) blot analysis. Therefore, we applied the sensitive technique of RT-PCR for analysis of expression. Primers were chosen to detect mRNA independent of alternative splicing (49). As a control, we compared the relative expression of the housekeeping GAPDH gene. GM-CSF receptor α was expressed in the myeloid cell lines U937, HL-60, Monomac, and NB4 and also in monocytes isolated from peripheral blood but not in the B-lymphoblastic cell line Raji or the T-lymphoblastic cell lines REX and Jurkat (Fig. 2A). These data confirm the myeloid cell-specific expression of the GM-CSF receptor α mRNA in the hematopoietic system.

The promoter of GM-CSF receptor α exhibits cell-type-specific activity. To analyze whether the promoter exhibits myeloid cell-specific activity, a DNA fragment spanning from bp −393 to +49 of the human GM-CSF receptor α gene (Fig. 1) was cloned into the luciferase reporter vector pXP2 to form -393GM-CSFr-luc. Promoter activity was determined by the luciferase assay after transfection into the human myelomonocytic cell line U937 and compared with the activity of transfected nonmyeloid cell lines, including the B-lymphoblastoid Raji and BJAB and T-lymphoblastoid Rex and Jurkat cell lines (Fig. 2B). All luciferase values were corrected for transfection efficiency by cotransfection of CMV-hGH and compared with those for the promoterless vector pXP2. In the myelomonocytic cell line, the GM-CSF receptor α 5′-flanking region demonstrated very strong activity. The luciferase activity of -393GM-CSFr-luc was 303-fold (mean) greater than that of pXP2. Comparative analysis revealed that this promoter is five- to eight-fold more active than in the nonmyeloid cells (Fig. 2B). These results demonstrate that the 5′-flanking region of the GM-CSF receptor α contains cell-type-specific activity.

The GM-CSF receptor α promoter contains an important functional site between bp −53 and −41. To localize the functionally important sites for the promoter activity in myeloid cells, we performed a deletion analysis of the 5′-flanking region of the GM-CSF receptor α gene. We created a series of deletions of the 5′-flanking region (Fig. 1 and 3), and the activities of the 5′ deletion series with endpoints at bp −278, −146, −70, −53, −41, and −10 were assessed in U937 cells by transient transfections (Fig. 3). Deletion to position −70 was of no significant consequence. Deletion to position −53 resulted in an increase of activity (mean, 2.3-fold). Additional deletion to position −41, which removed a potential PU.1 binding site, diminished the GM-CSF receptor α promoter activity from 668-fold above that for pXP2 to 88-fold, an 87% drop in activity. Further deletion to position −10 did not change the basal activity of the promoter.

PU.1 binds to the GM-CSF receptor α promoter region. The functionally important region between bp −53 and −41 contains a purine-rich sequence resembling sites known to bind PU.1 in myeloid cells (Fig. 1) (35). PU.1 is a myeloid and B cell-specific transcription factor which has been implicated in the transcriptional control of several myeloid cell-specific genes, including another receptor for a myeloid growth factor, the M-CSF receptor (70). Therefore, an oligonucleotide spanning the region from bp −53 to −37 was prepared and used in EMSA with either in vitro-translated PU.1 or nuclear extracts from U937 cells. As shown in Fig. 4, in vitro-translated PU.1 protein comigrates with a complex (labeled PU.1) formed with nuclear extracts from U937 cells. This complex can be inhibited by the wild-type PU.1 site (Fig. 4, lanes 4 and 9), but not by a mutant PU.1 site (Fig. 4, lanes 5 and 10; see also Fig. 5, lane 1). The complex can be supershifted by using a specific amino-terminal peptide antibody (Fig. 4, lanes 7 and 11). In vitro-translated Spi-B, a member of the ets family of transcription factors closely related to PU.1 (51), can also bind to this PU.1 site (data not shown). However, other ets family members, including ets-1, ets-2, elf-1, and fli-1, failed to interact with this site (data not shown).

In addition to the complex designated PU.1 in Fig. 4, faster- and more slowly migrating specific complexes can be observed in nuclear extracts of U937 cells. The faster-migrating band, indicated in Fig. 4 with an asterisk, has been observed previously in EMSA with probes containing PU.1 sites in the CD11b and the M-CSF receptor promoters and has been described extensively (6, 41, 70). This complex is formed by a proteolytic product of PU.1 which retains the carboxyl-terminal DNA binding domain but has lost the amino-terminal region recognized by the peptide antiserum. The more slowly migrating complex (PU-SF) can be diminished by both an antiserum raised against an amino-terminal PU.1 peptide (24, 46) and an antibody raised against the full-length PU.1 protein (54), indicating that PU.1 is involved in the interaction with another protein, which we refer to as PU.1-shifting factor, in the formation of this complex, PU-SF (Fig. 4, lane 11; Fig. 5, lanes 9 and 10). Furthermore, unlabeled oligonucleotides containing the PU.1 binding sites from the promoters of the CD11b and the M-CSF receptor genes inhibit the more slowly migrating complex, PU-SF, supporting the notion that it contains PU.1 (Fig. 5, lanes 2 and 4). While these functional PU.1 sites can compete for the formation of this complex, a purine-rich potential PU.1 site (35) from the GM-CSF receptor promoter (positions −10 to +7; Fig. 1) fails to compete (Fig. 5, lane 5),

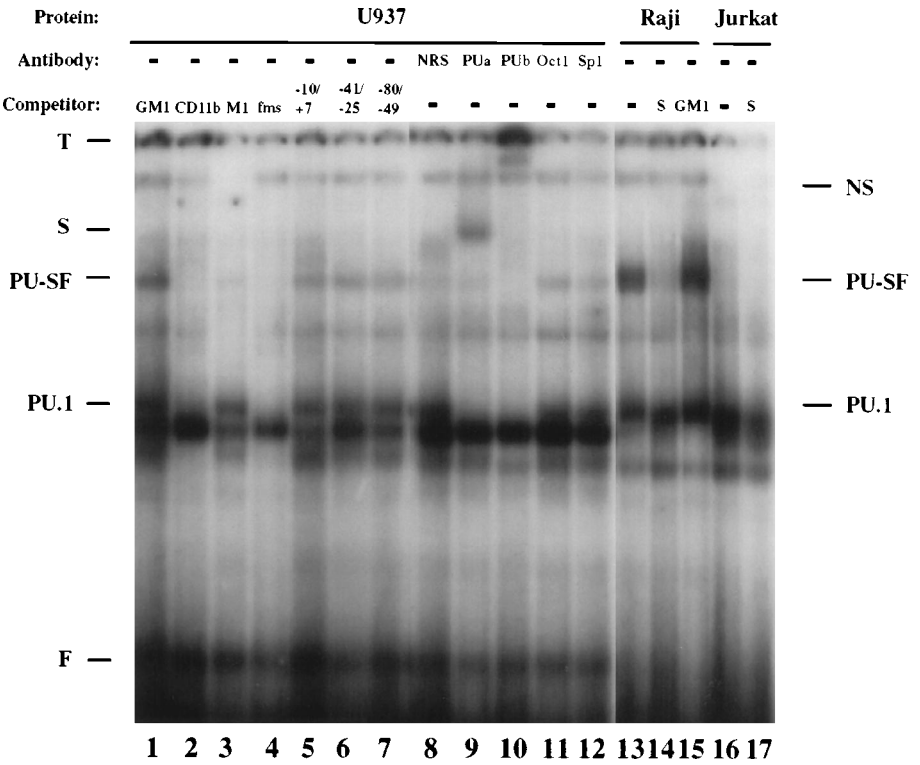


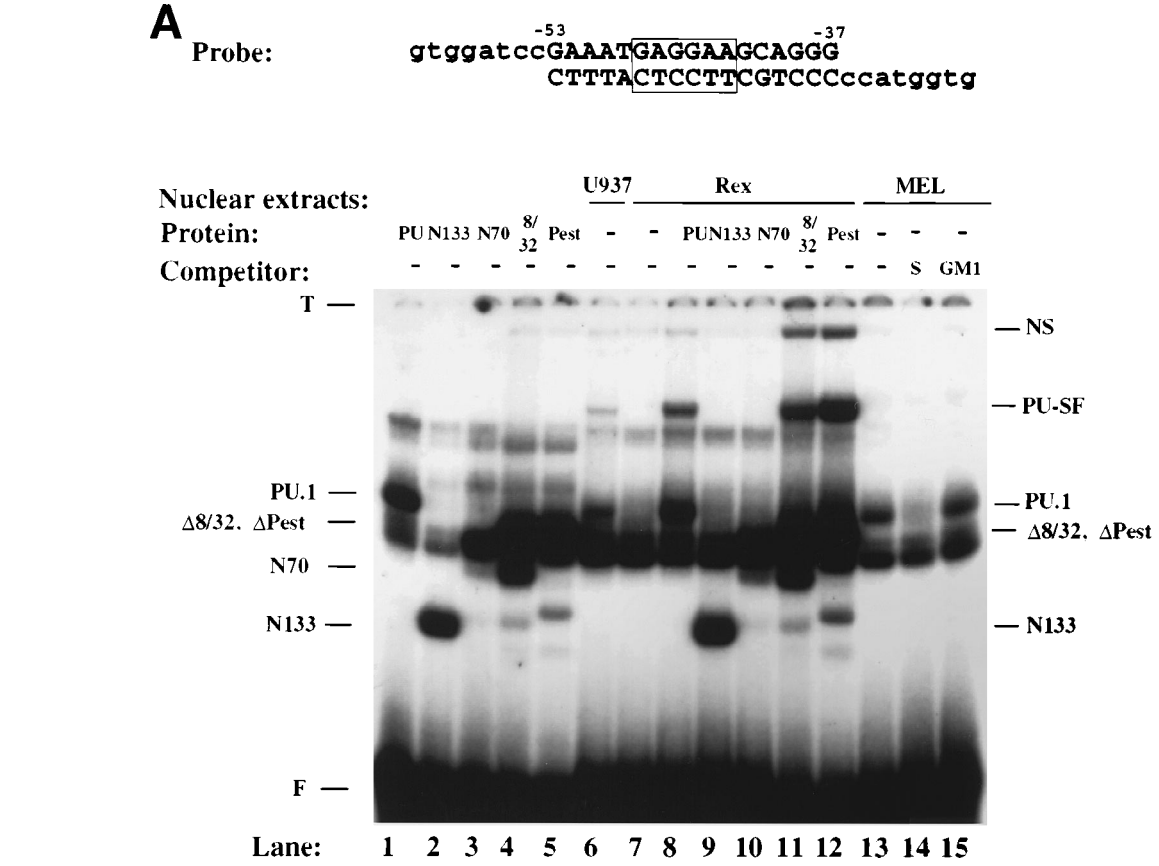
FIG. 5. Characterization of the PU-SF complex by gel mobility shift assay. The GM-CSF receptor α promoter oligonucleotide from position −53 to −37 (same as for Fig. 4) was used as a probe and incubated with 10 μg of nuclear extract prepared from either U937 cells (lanes 1 through 12), Raji B cells (lanes 13 through 15), or Jurkat T cells (lanes 16 and 17). The following unlabeled double-stranded competitor oligonucleotides were added to the reaction mixtures at a 100-fold molar excess: GM-CSF receptor α promoter bp −53 to −37 (self) (lanes 14 and 17), mutated PU.1 site (GM1) GM-CSF receptor α promoter (lanes 1 and 15), PU.1 binding site from the CD11b promoter (41) (lane 2), mutated PU.1 site from the CD11b promoter (M1 mutation [41]) (lane 3), M-CSF receptor (c-*fms*) promoter PU.1 binding site (70) (lane 4), GM-CSF receptor α promoter bp −10 to +7 (lane 5), GM-CSF receptor α promoter bp −41 to −25 (lane 6), and GM-CSF receptor α promoter bp −80 to −49 (lane 7). The following antisera were added as described in Materials and Methods: antiserum recognizing the amino terminus of PU.1 (lane 9), antiserum against the full-length PU.1 protein (lane 10), antiserum against Oct-1 (lane 11), antiserum raised against Sp1 (lane 12), and normal rabbit serum (lane 8). Abbreviations are as in Fig. 4; in addition, a nonspecific band observed near the top of the gel is labeled NS. The autoradiograph was extensively exposed in order to show the PU-SF complex in U937 cells.

indicating that it does not bind PU.1. Moreover, oligonucleotides spanning the regions adjacent to the PU.1 site in GM-CSF receptor α fail to compete for the formation of this complex (positions −41 to −25 [Fig. 5, lane 6]; positions −80 to −49 [Fig. 5, lane 7]), suggesting that this complex is not formed with a neighboring DNA-binding protein, particularly not with C/EBP, which binds to the upstream area (positions −80 to −49), as shown below.

PU.1 forms a novel complex via its amino-terminal domain. To further investigate which protein could be involved in the formation of PU-SF, we tested nuclear extracts from different sources. Nuclear extracts from the B-lymphoblastoid cell line Raji also contain PU.1 and form complexes with the GM-CSF receptor α PU.1 site similar to those found in U937 nuclear extracts, except that relatively more PU-SF (compared with full-length PU.1) is observed (Fig. 5, lane 13). Nuclear extracts from the T-lymphoblastoid cell lines Jurkat and REX, which do not contain PU.1, fail to form any specific complex (Fig. 5, lane 16; Fig. 6A, lane 7). However, when in vitro-translated PU.1 is added to PU.1-negative nuclear extracts from REX cells, a complex similar to that seen in myeloid and B cells is formed (Fig. 6A, lane 8). Moreover, nuclear extracts from a variety of cell lines, including HepG2 hepatoma cells (Fig. 6B, lane 8), Jurkat T cells and cervical carcinoma HeLa cells (data not shown), the glial cell line U87 (Fig. 6B, lane 4), and monkey kidney Cos cells (Fig. 6B, lane 10) are capable of forming PU-SF with in vitro-translated PU.1. However, the erythroid cell lines MEL, which overexpresses PU.1, and CB5, which does not express PU.1 (58), fail to form the PU-SF complex with in vitro-translated PU.1 (Fig. 6A, lane 13; Fig. 6B, lane 2). We therefore conclude that a protein which is not restricted to the cell types in which PU.1 is expressed is involved in the formation of PU-SF. Interestingly, Spi-B is also capable of forming the larger PU-SF complex (data not shown).

To localize the domain of the PU.1 protein which interacts with this protein, we in vitro translated a series of PU.1 proteins with deletions in the amino terminus. These truncated proteins retained the DNA binding domain located in the carboxyl-terminal sequence (residues 167 to 255) and were therefore capable of binding to the PU.1 site (Fig. 6A, lanes 1 to 5). We localized the region in the PU.1 protein involved in forming PU-SF to amino acids 32 to 70 in the transactivation domain: removal of either 133 or 70 amino acids from the amino terminus abolished the ability of PU.1 to form PU-SF (Fig. 6A, lanes 9 and 10). However, deletion of amino acids 8 through 32 in the amino terminus did not abolish the formation of the complex (Fig. 6A, lane 11).

We investigated whether PU-SF involved TBP or RB protein, ubiquitously expressed proteins which have been previously shown to interact with the amino terminus of PU.1 in vitro (19). The expression pattern of PU-SF (not in erythroid cells) made it unlikely to be TBP or RB protein, and an anti-



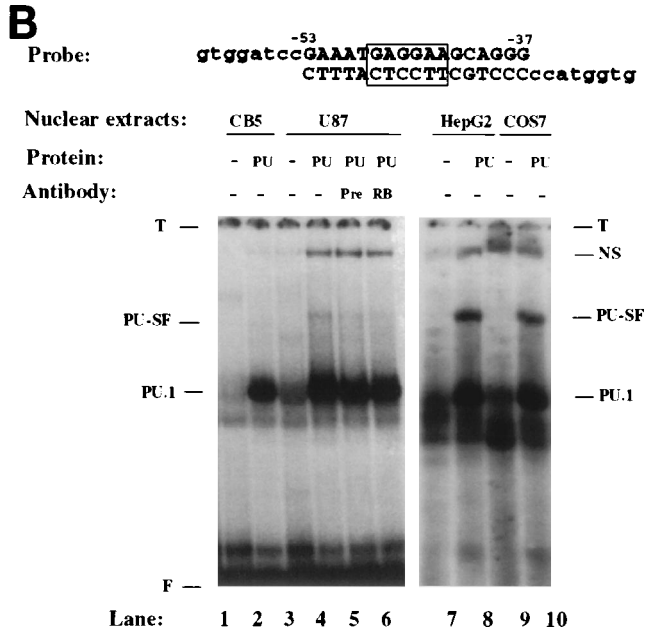


FIG 6. (A) PU.1 interacts via its amino terminus with another protein to form complex PU-SF. The GM-CSF receptor α promoter oligonucleotide spanning positions −53 to −37 was incubated either in the absence (lanes 1 through 5) or in the presence (lanes 7 through 12) of 10 μg of nuclear extract from REX T cells with the following in vitro-translated proteins: full-length PU.1 (lanes 1 and 8), N133 PU.1 (133 amino acids deleted from the amino terminus) (lanes 2 and 9), N70 PU.1 (70 amino acids deleted from the amino terminus) (lanes 3 and 10), Δ8/32 PU.1 (amino acids 8 to 32 deleted) (lanes 4 and 11), and ΔPest PU.1 (amino acids 119 to 160 deleted) (lanes 5 and 12). For comparison, the probe was incubated with 10 μg of nuclear extract from U937 cells (lane 6). In lanes 13 to 15, the probe was incubated with 10 μg of nuclear extract from MEL cells (MEL is an erytholeukemia line which overexpresses PU.1 because of viral insertional activation [33, 55]). The following unlabeled competitor oligonucleotides were added at a 100-fold excess to the reaction mixture: GM-CSF receptor α promoter bp −53 to −37 (self) (lane 14) and mutated PU.1 site GM-CSF receptor α promoter (GM1) (lane 15). All abbreviations are as in Fig. 5. In addition, on the left are indicated the positions of the complexes formed by in vitro-translated proteins Δ8/32 and ΔPest PU.1, which migrate just ahead of PU.1; N70, which migrates in a position similar to that of a nonspecific band (compare lanes 3 and 14); and N133 PU.1. The autoradiograph was heavily exposed to show the PU-SF complex in U937 cells (lane 6), as well as the absence of such a complex in MEL cells (lane 13). (B) Addition of PU.1 to nuclear extracts results in formation of PU-SF. The GM-CSF receptor α promoter oligonucleotide bp −53 to −37 was incubated with 10 μg of nuclear extract from the erythroid cell line CB5 (lanes 1 and 2), the glial cell line U87 (lanes 3 through 6), the hepatoma cell line HepG2 (lanes 7 and 8), and the kidney cell line Cos7 (lanes 9 and 10) in the absence (lanes 1, 3, 7, and 9) or presence (lanes 2, 4, 5, 6, 8, and 10) of 1 μl of in vitro-translated PU.1. In lane 6, 1.5 μl of an antiserum raised against RB protein was added to the reaction mixture; in lane 5, 1.5 μl of preimmune serum (Pre) was added to the reaction mixture. All other abbreviations are as in Fig. 5. The autoradiograph was heavily exposed to show the PU-SF complex in U87 cells (lanes 4 to 6) and the lack of such a complex in CB5 cells.

serum to TBP did not disrupt the PU-SF complex (data not shown). An antiserum raised against RB protein also did not alter PU-SF (Fig. 6B, lane 6). We also think it unlikely that PU-SF represents a complex formed by PU.1 and NF-EM5, since (i) the latter is reported to be a B cell-specific factor (46, 47), in contrast to PU-SF, which was formed with nuclear extracts from many cell types (other than erythroid cells) if PU.1 was present or added to the extracts (Fig. 5 and 6 and data not shown); (ii) deletion of the PEST domain (amino acids 119 to 160), which includes the site interacting with

TABLE 1. Formation of the PU-SF complex[a]

Derivation of probe (reference)	Probe no.	Sequence	PU-SF formation
GM-CSF receptor α promoter			
	1	gtggatccGAAATGAGGAAGCAGGG CTTTACTCCTTCGTCCCcatggtg	Yes
	2	TGAAATGAGGAAGCAGGG CTTTACTCCTTCGTCCCC	No
	3	gtggatccGAAATGAGGAAGCAGGG CTTTACTCCTTCGTCCCC	Yes
	4	TGAAATGAGGAAGCAGGG CTTTACTCCTTCGTCCCcatggtg	Yes
	5	gtggatccGAAATGAGGAAGCAGGGgtacca acctaggCTTTACTCCTTCGTCCCcatggtg	No
	6	AATCCTATGAAATGAGGAAGCAGGGGAGGGAG TAGGATACTTTACTCCTTCGTCCCCTCCCTCC	No
	7	TGAAATGAGGAAGCAGGG CTTTACTCCTTCGTCCCCTCCCTCC	Yes
	8	AATCCTATGAAATGAGGAAGCAGGG CTTTACTCCTTCGTCCCC	Yes
	9	AATttTATGAAATGAGGAAGCAGGG CTTTACTCCTTCGTCCCC	Yes
	10	gggggggGAAATGAGGAAGCAGGG CTTTACTCCTTCGTCCCC	No
	11	gagagagaGAAATGAGGAAGCAGGG CTTTACTCCTTCGTCCCC	No
	12	tcgagGAAATGAGGAAGCAGGGGg cCTTTACTCCTTCGTCCCCcagct	No
M-CSF receptor promoter (70)	13	tcgaCCTAGCTAAAAGGGGAAGAAGAGGATCAGC GGATCGATTTTCCCCTTCTTCTCCTAGTCGagct	No
	14	gtggatccTAAAAGGGGAAGAAGAG ATTTTCCCCTTCTTCTCccatggtg	Yes
CD11b promoter (41)	15	gtggatccGAAAAGGAGAAGTAGGA CTTTTCCTCTTCATCCTccatggtg	Yes
	16	GGCAGAAAAGGAGAAGTAGGAGGCA CGTCTTTTCCTCTTCATCCTCCGTC	No
	17	gctCAAAAGAAGGGCAGAAAAGGAGAAGTAG CGTCTTTTCCTCTTCATCCTCCGTC	Yes
	18	gctCAAAAGAAGGGCAGAAAAGGAGAAGTAG GTTTTCTTCCCGTCTTTTCCTCTTCATCCga	No
G-CSF receptor promoter (62)	19	cgggatccGGTTTCAGGAACTTCTCTTG CCAAAGTCCTTGAAGAGAACccatgggc	Yes

[a] Oligonucleotide probes with PU.1 binding sites were end labeled and assayed by EMSA to determine the DNA sequences involved in the formation of the PU-SF complex. Each probe was compared with the one shown in Fig. 4, 5, 6, 7, and 9 (probe 1 in this table), which was included on each gel as a standard, and each was subjected to competition with a 100-fold excess of unlabeled self competitor to demonstrate specificity. (See Fig. 7 as an example.) Probes 2, 5, 7, 8, 9, 11, 14, and 15 were also tested with a 100-fold excess of unlabeled non-self competitor (E18 ets consensus site, 5′-GATCGAGACCGGAAGTGGGG-3′), which did not compete for PU.1 or PU-SF complex formation. Uppercase letters represent promoter (genomic) sequences, while lowercase letters represent nonpromoter sequences. The sequences are aligned relative to the core PU.1 binding sites, as defined by methylation interference studies, as well as the string of three or four adenosine residues which are just 5′ of the GA core.

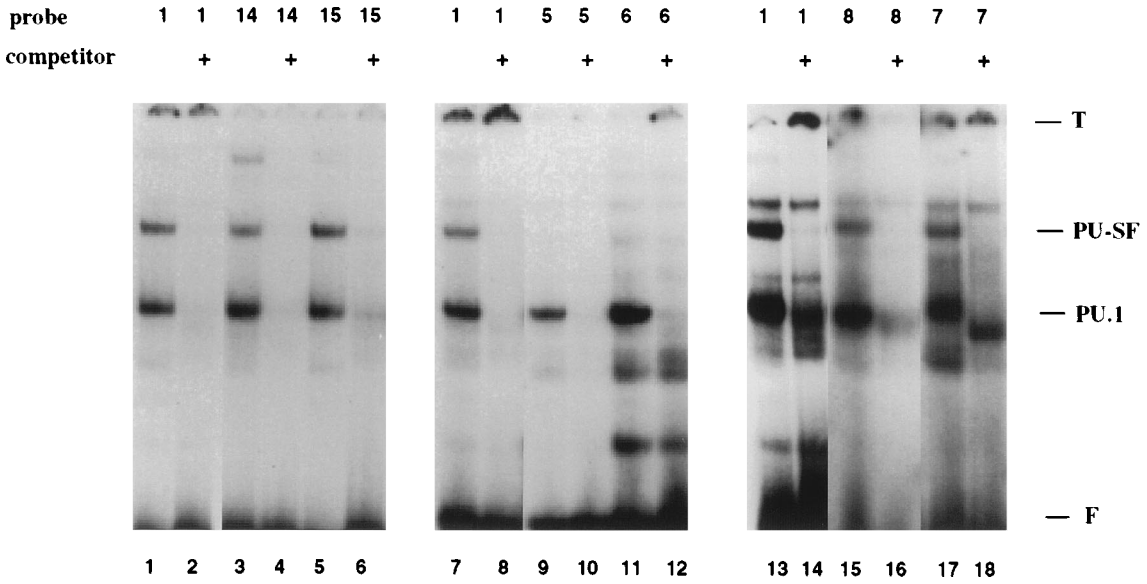


FIG. 7. DNA sequence requirements for the binding of PU-SF. Various oligonucleotide probes were analyzed for their abilities to bind PU-SF by EMSA. The probe number indicated at the top corresponds to the probe number in Table 1, where the sequences are listed. All probes were incubated with both in vitro-translated PU.1 and nuclear extracts from the Rex cell line, and in the even-numbered lanes they were inhibited with a 100-fold molar excess of unlabeled oligonucleotide. Lanes 1, 2, 7, 8, 13, and 14, GM-CSF receptor α probe, bp −53 to −37, with single-stranded flanking *Bam*HI and *Kpn*I sites (Table 1, probe 1). Competition with the same oligonucleotide, unlabeled, is shown in lanes 2, 8, and 14. Lanes 3 and 4, M-CSF receptor probe, bp −53 to −37, with single-stranded flanking *Bam*HI and *Kpn*I sites (Table 1, probe 14). Lanes 5 and 6, CD11b probe, bp −27 to −11, with single-stranded flanking *Bam*HI and *Kpn*I sites (Table 1, probe 15). Lanes 9 and 10: GM-CSF receptor α, bp −53 to −37, with double-stranded flanking *Bam*HI and *Kpn*I sites (Table 1, probe 5). Lanes 11 and 12, GM-CSF receptor α, bp −61 to −30 (Table 1, probe 6). Lanes 15 and 16: GM-CSF receptor α, bp −53 to −37, with an 8-base single-stranded 5′ overhang on the coding strand (Table 1, probe 8). Lanes 17 and 18, GM-CSF receptor α, bp −53 to −37, with an 8-base single-stranded 5′ overhang on the noncoding strand (Table 1, probe 7). Abbreviations are as in Fig. 5.

NF-EM5 (47), did not abolish the ability of PU.1 to form PU-SF (Fig. 6A, lane 12); and (iii) the PU-SF complex migrated with a mobility distinctly slower than that formed by a double-stranded oligonucleotide containing the PU.1/NF-EM5 site from the κ 3′ enhancer (46) (data not shown). We further asked whether this interaction is specific for the GM-CSF receptor α promoter PU.1 site or whether other functional PU.1 sites in myeloid cells can form this complex. In experiments performed simultaneously and run on the same gel, certain oligonucleotides containing the PU.1 site from the M-CSF receptor (70) or the CD11b promoter (41) failed to form PU-SF when incubated with U937 nuclear extracts (Table 1, probes 13, 16, and 18).

Formation of PU-SF requires a single-stranded pyrimidine region. The failure of certain M-CSF receptor or CD11b PU.1 site oligonucleotides to form a complex with PU-SF prompted us to investigate whether specific DNA sequences were required for such an interaction. Methylation interference assays did not indicate any significant difference between the PU.1- and PU-SF-bound probe (data not shown). We then designed a series of oligonucleotide probes which we tested by EMSA with in vitro-translated PU.1 and nuclear extracts from the REX cell line, a combination which produced a large quantity of PU-SF-DNA complex (Fig. 6A, lane 8).

Interestingly, PU-SF formation was lost when both single-stranded flanking *Bam*HI (coding strand) and *Kpn*I (noncoding strand) sites were eliminated from the GM-CSF receptor α probe, but it was regained when either site was added back (Table 1, probes 1 to 4) or when those sequences were added to the M-CSF receptor and CD11b PU.1 binding sites (Fig. 7, lanes 3 to 6. In addition, we could not detect PU-SF when using a probe with double-stranded flanking *Bam*HI and *Kpn*I sites (Fig. 7, lanes 9 and 10) or when using a double-stranded probe which extended the GM-CSF receptor α sequence to an equivalent length (Fig. 7, lanes 11 and 12). However, 8-base single-stranded 5′ overhangs of GM-CSF receptor α sequence on either the coding or noncoding strand were sufficient to allow the formation of the complex (Fig. 7, lanes 15 to 18).

We then asked whether the requirement of PU-SF for single-stranded DNA was sequence specific. Changing the cytosine residues at bp −57 and −58 of the GM-CSF receptor α 5′ overhang to thymidines did not prevent the formation of the complex. However, PU-SF could not be detected when the single-stranded 5′ overhangs were constructed entirely of purines (Table 1, probes 9 to 11). These data indicate that the formation of PU-SF has some requirement for pyrimidines as well as for single-stranded DNA.

Single-stranded oligonucleotides (Table 1, probes 1, 7, and 8) could not inhibit either the PU.1 or the PU-SF complexes, nor could an oligonucleotide (GM1 [see Fig. 9]) mutated only at the PU.1 binding site (data not shown). However, an oligonucleotide which, as a probe, could bind PU.1 but not PU-SF (Table 1, probe 12) did inhibit both complexes (data not shown). These observations are consistant with the hypothesis that PU-SF requires PU.1 to bind DNA. The single-stranded or mutated competitor is unable to bind PU.1 and therefore unable to bind PU-SF, and a competitor that binds PU.1 but not PU-SF will block PU.1 from the probe and therefore prevent binding to PU-SF indirectly.

Mutation of the PU.1 site abolishes binding of PU.1 and decreases promoter activity in myelomonocytic cells. To define the residues in the GM-CSF receptor α promoter contacted by the PU.1 protein, methylation interference assays were performed with a DNA fragment including the region between positions −70 and +49. As shown in Fig. 8, the PU.1 protein directly contacts the three G residues at positions −48, −46, and −45. No other strong contacts could be observed, indicating that this site is the only PU.1 binding site in the proximal

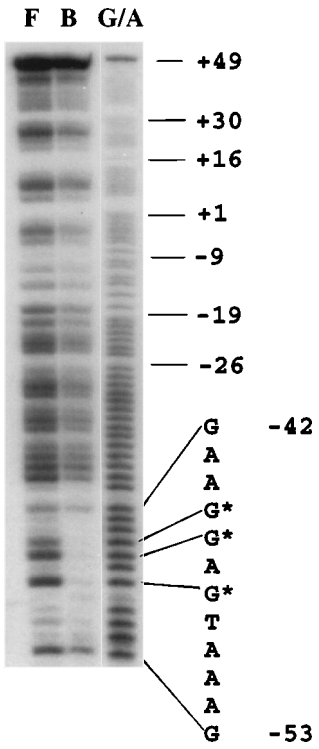


FIG. 8. Methylation interference analysis of DNA contact points of PU.1 to the GM-CSF receptor α promoter. The GM-CSF receptor α promoter fragment from bp −70 to +49 was end labeled on the 5′ end of the noncoding strand and methylated by treatment with limiting quantities of dimethyl sulfate. Free DNA (lane F) and DNA from the complex formed by in vitro-translated PU.1 with the bp −70 to +49 fragment (lane (B) was subjected to piperidine-mediated cleavage of methylated guanosine residues. Lane G/A, adenosine-guanosine sequence standards were prepared from the unmethylated probe by a chemical cleavage protocol (30). The sequence of the region between bp −53 and −42 is shown on the right. The asterisks indicate the direct contacts between the DNA and PU.1 at residues −45, −46, and −48.

promoter, since PU.1 also does not bind to oligonucleotides extending upstream to position −80 and downstream to +7 (Fig. 5, lanes 5 to 7).

To verify the importance of PU.1 for the activity of the GM-CSF receptor α promoter, the three guanosine residues identified by methylation interference to be contacted by PU.1 were mutated into cytosine residues (Fig. 9). An oligonucleotide with the mutated PU.1 site could not compete for PU.1 binding to the wild-type region from bp −53 to −37 (Fig. 4, lanes 5 and 10; Fig. 5, lane 1). Furthermore, the same PU.1 mutant site oligonucleotide did not bind to PU.1 either as in vitro-translated protein or U937 nuclear extracts in gel shift assays (Fig. 9, lanes 3 and 7). The mutant oligonucleotide also was not able to form PU-SF observed with nuclear extracts, supporting the idea that DNA binding of PU.1 is critically involved in the formation of this complex (Fig. 9, lane 7). Additionally, no specific protein binding site was created by the mutation (Fig. 9, lane 7). In nuclear extracts from U937 cells, we observed only a nonspecific band (Fig. 9, lanes 7 to 10) which comigrated with a similar nonspecific band seen with the wild-type oligonucleotide (Fig. 9, lane 15) at a position slightly lower than that of PU.1 (Fig. 9, lanes 11, 13, and 14). This same mutation was then introduced into the GM-CSF receptor α promoter-luciferase construct. We introduced the mutation both into -70GM-CSFr-luc, which retains full promoter activity compared with -393GM-CSFr-luc, and into -53GM-CSFr-luc, which has increased activity (Fig. 3). Comparative analysis of transient transfections in U937 cells revealed that the promoter activities of both mutants were significantly decreased compared with that of the wild type (Fig. 10). -53GM-CSFr(GM-1)-luc, containing mutations of the PU.1 site at the three base pairs which contact PU.1, demonstrated 31% of the activity of the wild type (Fig. 10A). The activity of the -70GM-CSFr(GM-1)-luc construct was reduced to 13% of that of the wild-type promoter (Fig. 10B). In nonmyeloid Jurkat cells, the activity of -70GM-CSFr-luc was only 8% of the activity in U937 cells, and mutation of the PU.1 site (-70GM-CSFr(GM-1)-luc did not result in a significant change of promoter activity (Fig. 10B). These data indicate the myeloid specificity and functional importance of PU.1 for the regulation of GM-CSF receptor α.

C/EBPα binds to the GM-CSF receptor α promoter. Deletion analysis of the GM-CSF receptor α promoter identified a fragment between residues −70 and −54 whose deletion resulted in an increase in promoter activity (Fig. 3). We asked whether proteins involved in negative regulation or involved in a competition between a positive and a negative factor could bind to this region. A CCAAT site around bp −60 represents a potential protein binding site in this fragment. CCAAT sites are target sequences for a variety of both general and cell-type-specific transcription factors (20). C/EBPs are involved in the control of cell-type-specific differentiation and proliferation, and the α, β, and δ forms are expressed specifically in myeloid cells (36, 57). Theses factors could be involved in myeloid cell proliferation and differentiation through the transcriptional control of the expression of growth factor receptors, like GM-CSF receptor α, that play a crucial role in this process.

We therefore analyzed whether this region can bind C/EBP. An EMSA with a nuclear extract from U937 cells and an oligonucleotide spanning the GM-CSF receptor α promoter from position −80 to −49 showed binding of a specific complex (complex C/EBPα; Fig. 11, lane 2), which could be blocked by an excess of unlabeled self oligonucleotide (Fig. 11, lane 3), by a shorter oligonucleotide spanning positions −70 to −54 and retaining the CCAAT region (Fig. 11, lane 4), and by an oligonucleotide containing a C/EBP site from the M-CSF receptor (c-*fms*) (Fig. 11, lane 6) (69, 71). However, an ets consensus binding oligonucleotide which binds multiple ets factors (23, 66) could not inhibit the formation of this complex (Fig. 11, lane 5). The specific complex could be diminished by a rabbit antiserum raised against the carboxyl-terminal four-fifths of C/EBPα (Fig. 11, lane 8) but not by normal rabbit serum (Fig. 11, lane 7) or antisera against other C/EBP family members (Fig. 11, lanes 9 and 10). Besides the C/EBPα complex, we could identify faster-migrating specific complexes (Fig. 11, complexes B and C). These complexes could be observed as well in Cos7 cells (Fig. 12) and may therefore represent non-cell-type-specific activities. Competition studies with a 100-fold molar excess of either unlabeled strand of the probe indicated that complexes B and C bind single-stranded DNA (data not shown). Single-strand competitors containing sequences from bp −70 to −49 did not inhibit complexes B and C, while a single strand containing the sequence from bp −80 to −70 did, indicating that B and C bind the region from bp −80 to −70 of the probe when it is in a single-stranded form (data not shown).

To obtain further evidence that the complex is C/EBPα, we performed a gel shift with a nuclear extract from Cos7 cells which were transiently transfected with a C/EBPα expression plasmid (Fig. 12). C/EBPα formed a specific complex with the oligonucleotide from bp −80 to −49 (Fig. 12, lane 2), similar to that seen in the U937 cell nuclear extract (Fig. 11, lane 2), which could also be inhibited by an oligonucleotide spanning

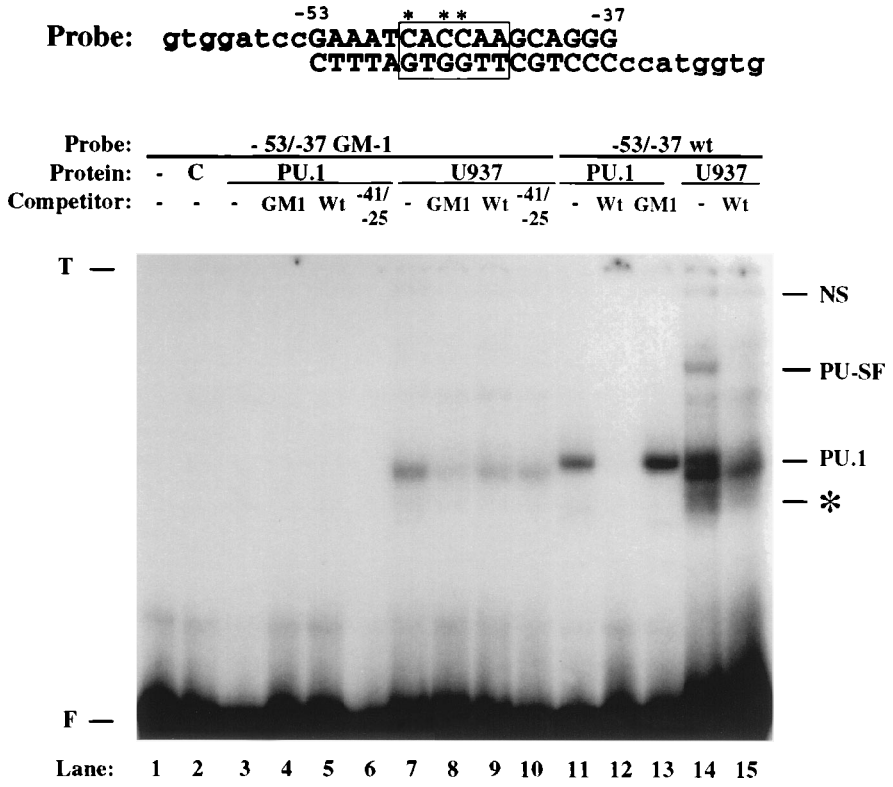


FIG. 9. Mutation of the PU.1 site abolishes PU.1 binding in EMSA. The probe used in lanes 1 to 10 was a double-stranded oligonucleotide spanning the GM-CSF receptor α promoter from position −53 to −37 with point mutations of the guanosine residues (bp −48, −46, and −45) identified by methylation interference to be involved in PU.1 binding (Fig. 8) (GM1). In lanes 11 to 15, the probe was the wild-type (wt) oligonucleotide used as a control. Probes were end labeled and incubated with either 1 μl of in vitro-translated PU.1 (lanes 3 to 6 and 11 to 13) or 10 μg of nuclear extract from U937 cells (lanes 7 to 10, 14, and 15). A 100-fold molar excess of the following unlabeled competitor oligonucleotides was added to the reaction mixtures: mutated PU.1 site GM-CSF receptor α promoter (GM1) (lanes 4, 8, and 13), wild-type GM-CSF receptor α promoter bp −53 to −37 (Wt) (lanes 5, 9, 12, and 15), and GM-CSF receptor α promoter bp −41 to −25 (lanes 6 and 10). All other abbreviations are as in Fig. 4 and 6. A nonspecific complex, which does not compete with excess cold self oligonucleotide (lane 9), migrates just ahead of PU.1 and is seen in lanes 7 to 10, 14, and 15 (also observed in Fig. 4 to 6).

positions −70 to −54 (data not shown) but not by an oligonucleotide in which the adenosine residues of the CCAAT site were mutated into guanosine residues (GM2) (Fig. 12, lane 4). Moreover, this complex could be inhibited by a high-affinity C/EBP binding site (Fig. 12, lane 6) (5, 60) but not by a mutated site (Fig. 12, lane 7). C/EBPα binding was abolished by the specific α antiserum (Fig. 12, lane 9), while antisera against C/EBPβ and C/EBPδ had no effect (Fig. 12, lanes 10 and 11). Gel shift assays with Cos7 cell extracts transfected with C/EBPβ and -δ resulted also in the formation of specific complexes with the oligonucleotide probe from bp −80 to −49 which appeared similar to complexes formed by these extracts with the high-affinity consensus C/EBP site (5, 60) (data not shown). However, the binding of C/EBPβ to the GM-CSF receptor α CCAAT site was much weaker than that observed with the consensus high-affinity C/EBP site (data not shown). Moreover, supershift experiments with antisera raised against C/EBPβ and -δ and the oligonucleotide from bp −80 to −49 did not result in supershifts in U937 nuclear extracts (Fig. 11, lanes 9 and 10). The faint complex (NS) suggesting a supershift can also be observed in Cos7 extracts transfected with C/EBPα (seen in the longer exposures of Fig. 12, lanes 10 and 11) but not in untransfected Cos7 cells, and it therefore most likely represents a cross-reaction of these C/EBP antibodies with C/EBPα. However, in U937 cells, we observed significant levels of C/EBPα and not C/EBPβ or C/EBPδ (Fig. 11) (71). From these results taken together, we conclude that homodimers of C/EBPα are the major C/EBP form in U937 cells binding to the GM-CSF receptor α CCAAT site.

Mutation of the CCAAT site abolishes binding of C/EBPα and diminishes promoter activity. To address the question whether this CCAAT site could be responsible for the negative regulatory effect of this area, we mutated this site by point mutations changing two adenosine residues at positions −60 and −61 into guanosines, which abolished the binding of C/EBP (Fig. 12, lane 4). We introduced this mutation to form the mutant construct -70GM-CSFr(GM-2)-luc. Comparative analysis of this construct in transient transfections in U937 cells revealed that this site is a positive regulatory site. Mutation of the CCAAT site diminished the activity to 30% of the wild-type activity in U937 cells (Fig. 10B). However, in nonmyeloid Jurkat cells the activity of -70GM-CSFr(GM-2)-luc was slightly greater than that of the wild-type construct, indicating the myeloid specificity of the positive function of the CCAAT site. These data point to a positive-negative role of the region from bp −70 to −54 in which C/EBPα acts as a positive regulator. Mutation of both the PU.1 and CCAAT sites had no additional effect above that of a single mutation of each site, pointing to an independent function of each site (results of two experiments; data not shown).

PU.1 and C/EBPα activate the GM-CSF receptor α promoter in nonmyeloid cells. To further establish the functional importance of PU.1 and C/EBPα, we performed transactivation experiments with the nonmyeloid cell line HepG2, which

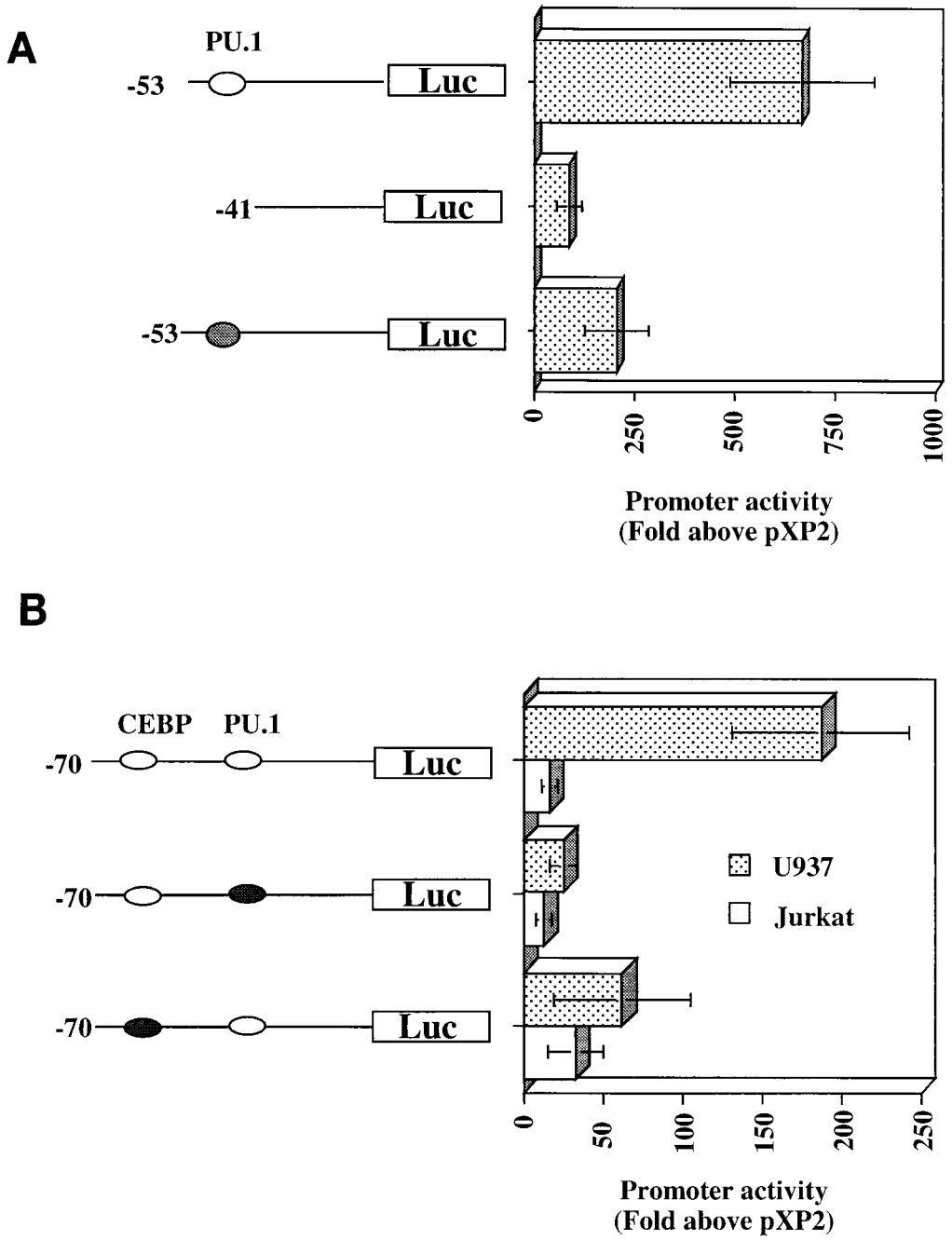


FIG. 10. Point mutations of the PU.1 and CCAAT sites diminish the GM-CSF receptor α promoter activity in a cell-type-specific way. (A) -53GM-CSFr(GM-1)-luc (bottom bar graph, shaded oval), extending from bp −53 to +49 and containing the mutated PU.1 site, was transfected into U937 cells, and the luciferase activity was compared with the activities of the wild-type -53GM-CSFr-luc (top bar, white oval) and the PU.1 site deletion -41GM-CSFr-luc (middle bar). The positions of the 5′ end points are indicated. (B) Wild-type -70GM-CSFr-luc (top bar graph, white ovals), mutated PU.1 site -70GM-CSFr(GM-1)-luc (middle bar, shaded oval representing the GM1 PU.1 site mutation and white oval indicating a wild-type CCAAT site), and mutated CCAAT site -70GM-CSFr(GM-2)-luc (bottom bar, shaded oval representing the GM2 CCAAT site mutation and white oval indicating a wild-type PU.1 site) were transiently transfected into myelomonocytic U937 and Jurkat T cells. Three independent experiments were performed, and the average fold luciferase activity over that with promoterless pXP2 and standard errors of the mean were calculated.

lacks endogenous PU.1 (Fig. 6B, lane 7) and expresses C/EBPα only at very low levels (14). -70GM-CSFr-luc was transfected into HepG2 cells along with either the full-length PU.1 expression plasmid (PU.1-pECE), the expression plasmids for the amino-terminally truncated form of PU.1 (N133 PU.1 and N70 PU.1), or the C/EBPα expression plasmid (MSV-C/EBPα). The activity of the GM-CSF receptor α promoter in the presence of cotransfected full-length PU.1 was increased to twice that of the control, while the presence of C/EBPα increased the promoter activity to 180% (Fig. 13A). In contrast, cotransfection with the amino-terminally truncated forms of PU.1 resulted in either a slight decrease (N133 PU.1) or no change (N70 PU.1) in promoter activity. The addition of PU.1 to C/EBPα did not potentiate transactivation of either

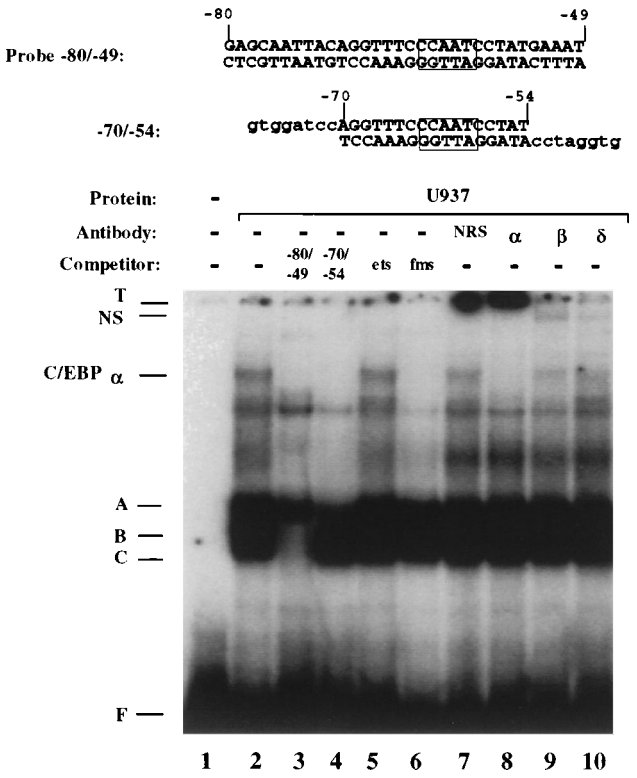


FIG. 11. Identification of C/EBPα binding to the GM-CSF receptor α promoter by gel mobility shift assay. A double-stranded GM-CSF receptor α promoter oligonucleotide extending from position −80 to −49 was end labeled and incubated in the absence (lane 1) or presence (lanes 2 through 10) of 10 μg of nuclear extract from U937 cells. A 100-fold molar excess of the following unlabeled competitor oligonucleotides was added to the reaction mixtures: GM-CSF receptor α promoter position −80 to −49 (self) (lane 3), GM-CSF receptor α promoter −70 to −54 (lane 4), E18 ets consensus site (see Table 1) (nonself) (lane 5) (23, 66), and M-CSF receptor (c-*fms*) promoter C/EBP site (lane 6). In lanes 7 to 10, 1.5 μl of antisera against C/EBPα (lane 8), C/EBPβ (lane 9), and C/EBP δ (lane 10) or normal rabbit serum (lane 7) were added to the reaction mixtures. Abbreviations: T, top of gel; NS, a nonspecific band which is observed in nonmyeloid cell extracts in the absence of C/EBPs and likely enhanced by the presence of the β or δ serum in lanes 9 and 10; C/EBPα, gel shifted complex formed by C/EBPα; A, nonspecific complex; B and C, specific single-stranded-DNA-binding complexes (shorter exposure of the autoradiograph reveals two bands; the autoradiograph was heavily exposed to show the C/EBPα complex). All other abbreviations are as in Fig. 6.

factor alone (data not shown). Furthermore, PU.1 was able to enhance the activity of a construct which contained a single PU.1 binding site upstream of the minimal TK promoter an average of sixfold (Fig. 13B). A plasmid with a mutation in the PU.1 binding site did not show transactivation greater than that of the minimal TK promoter. These results support the functional importance of C/EBPα and PU.1 in the regulation of GM-CSF receptor α expression. Moreover, it delineates a role for the amino-terminal domain of PU.1 in the activation of the GM-CSF receptor α promoter.

DISCUSSION

The expression of specific growth factor receptors is critically important for the development of hematopoietic cells, and therefore the factors regulating their expression could be key regulators of hematopoiesis. Recently PU.1 emerged as a candidate key regulator as suggested by gene inactivation and targeting studies (56, 66). Most myeloid cell-specific gene promoters, including the M-CSF receptor (70), contain functional PU.1 sites. The role of members of the C/EBP family of transcription factors for hematopoiesis is less well studied; only a few mammalian myeloid target genes have been definitely identified so far (3, 4, 36, 37, 48). Here, we demonstrate that the 5′-flanking region of the GM-CSF receptor α gene, which demonstrates cell-type-specific activity in myeloid cells, contains functionally important PU.1 and C/EBPα sites. While PU.1 and C/EBPα are also expressed outside the myeloid lineage, myeloid specificity could be obtained by the combination of both factors. PU.1, expressed in myeloid and B cells (6, 24), is critical for the myeloid activity of the GM-CSF receptor α promoter, as is C/EBP, which within the hematopoietic system is expressed specifically in myeloid cells and not in other lineages, such as B cells (57, 64).

In the region extending from position −80 to −49, we could detect specific DNA binding in U937 myeloid cells by C/EBPα but not by other C/EBP proteins. The mutation of two bases within this region (mutant GM2, containing mutations in the CCAAT site at bp −60 and −61), which abolished C/EBPα binding, decreased promoter activity by 70%, indicating that C/EBPα acts as a significant positive regulator of GM-CSF receptor α. However, two other complexes, found in both myeloid and nonmyeloid cells, bind to this region (complexes B and C; Fig. 11 and 12). These complexes do not bind to the C/EBP site (Fig. 11, lanes 4 and 5) as defined by the GM2

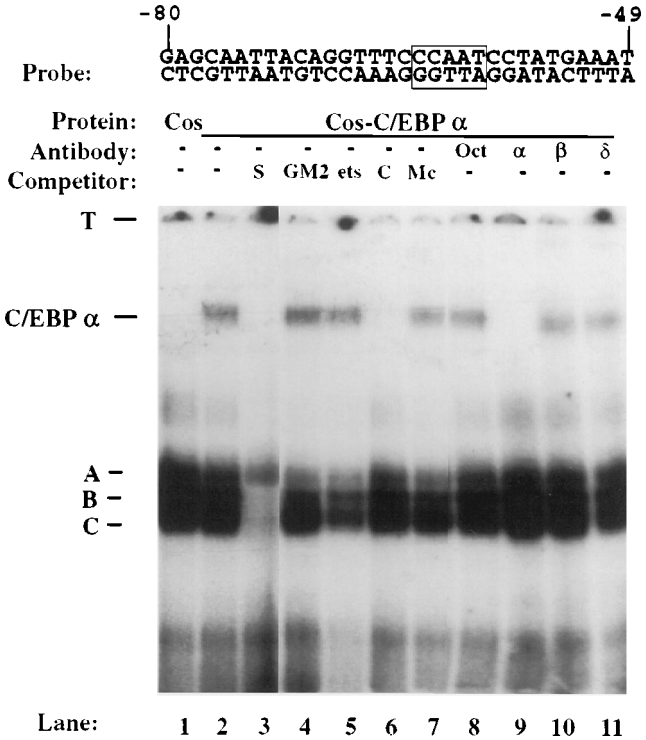


FIG. 12. Characterization of C/EBPα binding to the GM-CSF receptor α promoter in EMSA. The end-labeled GM-CSF receptor α promoter oligonucleotide extending from bp −80 to −49 was incubated with nuclear extract from mock-transfected Cos7 cells (lane 1) or from Cos7 cells transfected with a C/EBPα expression vector (lanes 2 through 11). The following unlabeled competitor oligonucleotides were added at a 100-fold molar excess to the reaction mixtures: GM-CSF receptor α promoter bp −80 to −49 (self) (lane 3), mutated CCAAT site GM-CSF receptor α promoter bp −73 to −54 (GM2) (lane 4), E18 ets consensus site (for sequence, see Table 1) (lane 5) (23, 66); high-affinity C/EBP binding site C (5′-TGCAGATTGCGCAATCTGCA-3′) (lane 6) (60), and mutated C/EBP binding site Mc (5′-TGCAGAGACTAGTCTCTGCA-3′) (lane 7) (60). In lanes 8 to 11, antisera against Oct-1 (lane 8), C/EBPα (lane 9), C/EBPβ (lane 10), and C/EBPδ (lane 11) were added to the reaction mixtures. Abbreviations are as described for Fig. 11.

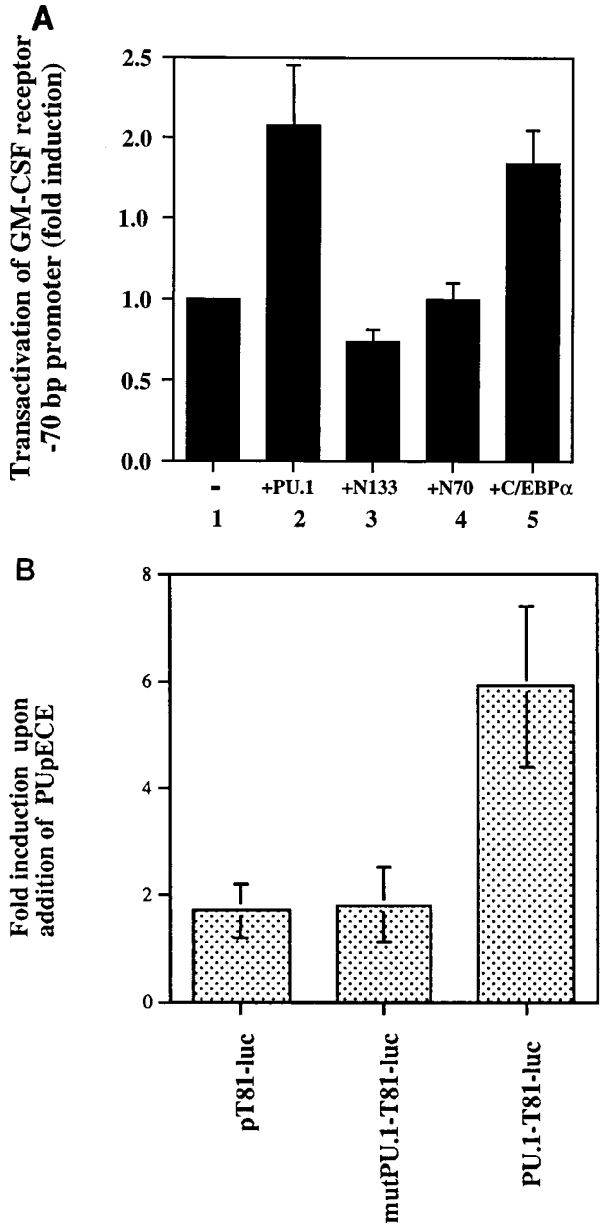


FIG. 13. Transactivation of the GM-CSF receptor α PU.1 site. (A) Transactivation of the GM-CSF receptor promoter in nonmyeloid cells. The hepatoma cell line HepG2 was transfected by the $Ca_3(PO_4)_2$ precipitation method with 10 μg of the GM-CSF receptor α promoter construct -70GM-CSFr-luc and with one of the following expression constructs: none (bar 1), the PU.1 expression construct PUpECE (+PU.1) (bar 2), an expression construct with amino acids 1 to 133 of PU.1 deleted (+N133) (bar 3), an expression construct with amino acids 1 to 70 of PU.1 deleted (+N70) (bar 4), and the C/EBPα expression construct MSV-C/EBPα (+C/EBPα) (bar 5). Luciferase was measured 40 h after transfection and normalized for transfection efficiency with the cotransfected growth hormone plasmid CMV-hGH. The data are the means of three independent experiments. The standard errors of the means are indicated by the error bars. (B) Transactivation of a minimal TK promoter containing the GM-CSF receptor α PU.1 site. CV-1 cells were transfected with either the parental TK-luciferase vector (pT81-luc), pT81-luc containing a single PU.1 site with the GM1 mutation (described in Materials and Methods and shown in Fig. 9) (mutPU.1-T81-luc), or pT81-luc containing a single wild-type PU.1 binding site, bp −53 to −36 (PU.1-T81-luc), with and without 1 μg of PUpECE. The results are expressed as the average fold increase in activity upon addition of PUpECE. The error bars represent the standard error of the mean of at least four experiments.

mutation (Fig. 12, lane 4) but can be inhibited by either unlabeled strand containing sequences from bp −80 to −70, suggesting that they are formed by single-strand-binding proteins which do not exhibit strand specificity.

Deletion of the region from bp −70 to −53 led to an increase in promoter activity in U937 cells (Fig. 3), indicating that this region mediates negative as well as positive regulatory effects. Competition by overlapping binding sites of a non-cell-type-specific factor with C/EBP could contribute to cell type specificity. An example for the model of competition by use of overlapping DNA binding sites between negative and positive factors has been described for the myeloid cell-specific gp91-phox promoter (61). Binding of the ubiquitously expressed CCAAT displacement protein results in repression of the gp91-phox gene in nonmyeloid and early myeloid cells (61). DNA binding studies with the CCAAT region of GM-CSF receptor α showed that CCAAT displacement protein does not bind to this region (data not shown). Another example for negative regulation is the conversion of a transcriptional activator into a repressor. The beta interferon gene, which is upregulated by viral induction, is switched off in uninduced cells by a repressor that first was believed to block the binding to an adjacent NF-κB site (17). This protein has been recently cloned in *Drosophila melanogaster* as the high-mobility-group (HMG)-like protein DSP1, which can switch NF-κB from a transcriptional activator to a repressor (27). In the case of GM-CSF receptor α, further studies are needed to identify the putative negative regulatory factor or factors, their mechanism of action, and their roles in the regulation of expression of the receptor.

We identified C/EBPα to be the major C/EBP form in myeloid extracts of U937 cells binding to the CCAAT site at position −61. This finding is consistent with previous protein expression data obtained by immunoblots (57). The relative expression of C/EBP proteins is temporally regulated during myeloid cell differentiation (36, 57). It is interesting to speculate that changes in the C/EBP expression pattern could influence the expression of GM-CSF receptor α. It is noteworthy that GM-CSF receptor α is upregulated early during IL-3-plus-GM-CSF-induced differentiation of $CD34^+$ progenitor cells and decreases later when the cells have undergone further maturation (data not shown). A similar pattern has been observed for protein expression of C/EBPα during differentiation of the murine myeloid progenitor cell line 32Dc13 (57). Other C/EBPα target genes in myeloid cells include the receptor for M-CSF (69, 71) and G-CSF (62), both of which are also regulated by PU.1 (62, 70). Therefore, we postulate a major role for C/EBPs in the specific expression of myeloid CSF receptor genes. It will be the focus of future research to elucidate the role of the C/EBPs in myeloid cell differentiation. GM-CSF receptor α is predicted by these studies to be a critical target for these transcription factors.

In gel shifts with the GM-CSF receptor α promoter PU.1 site, we observed the formation of a more slowly migrating complex (PU-SF) with nuclear extracts of myeloid and B cells. This complex could be formed by addition of nuclear extracts from a variety of cell types (but not from erythroid cells) to either PU.1 or the closely related ets family member Spi-B (51). We have not observed formation of the PU-SF complex when the PU.1 binding site is replaced by a C/EBP binding site, even when the oligonucleotide contains single-stranded ends (62a). Comparative analysis with studies of other myeloid PU.1 sites indicates that this complex is not unique for the GM-CSF receptor α PU.1 site. Our data suggest that the PU-SF complex is novel and does not involve proteins which have been previously shown to interact with PU.1: TBP (19), RB protein (19),

or NF-EM5 (11, 46, 47). The importance of the formation of PU-SF for the activity of the GM-CSF receptor α promoter was supported by transactivation of the promoter by full-length PU.1 but not by amino-terminally truncated forms of the PU.1 protein which fail to form PU-SF. In these studies, the magnitude of transactivation of GM-CSF receptor α by PU.1 was only twofold. However, the extent of transactivation does not necessarily reflect the functional importance of the PU.1 site, particularly when the transactivation studies are performed with the native promoter. Another promoter with a functionally critical PU.1 site which is only moderately transactivated (two- to threefold) by PU.1 is CD11b (41). Experiments performed with the PU.1 site placed upstream of a heterologous promoter show that the GM-CSF receptor α PU.1 site could be transactivated an average of sixfold.

EMSA studies indicate that DNA interaction with PU-SF requires, in addition to PU.1, a single-stranded region of DNA containing pyrimidines proximal to a PU.1 binding site. Although the inability of the amino-truncated forms of PU.1 protein to transactivate GM-CSF receptor α supports the hypothesis that PU-SF has a functional role in this promoter, we cannot yet exclude the possibility that those regions of the PU.1 protein are critical for an unrelated activating function. Additional studies will be required to establish the functional importance of the formation of PU-SF. There is substantial evidence for the role of single-stranded binding factors in other promoters, both as activators and repressors (31, 65, 67). In the simplest scenario, two factors which bind to opposite strands of the same site, VACssBF1 and VACssBF2, have been shown to play a role in repression of the vascular smooth muscle α-actin gene promoter, perhaps by stabilizing single-stranded structure and preventing the binding of a positive regulatory factor which requires double-stranded DNA (65). Other theories include stabilization of single-stranded regions that serve to relieve torsional stress induced by transcription or provide a flexible joint to facilitate DNA-bound protein-protein interactions (9). Wang et al. postulate that the binding of the serum response factor to the serum response element of the platelet-derived growth factor A-chain promoter induces DNA bending and strain which can be released by strand separation and that this altered conformation is stabilized by a second, single-stranded binding factor, SSBF (67). It is possible that the interaction between PU.1 and PU-SF provides a similar function. For example, PU.1 might recruit PU-SF to stabilize an alternative DNA conformation.

The domain of the PU.1 protein involved in the formation of PU-SF is located in the amino terminus between amino acids 32 and 70. Other PU.1 targets in which the amino-terminal region of the protein is essential include the J chain gene in B cells (59) and the IL-1β promoter in myeloid cells (25). Further localization of the domain in the PU.1 protein responsible for the formation of PU-SF and identification of the factor involved in PU-SF formation will help to define the specific role of PU-SF in GM-CSF receptor α expression.

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