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The Myeloid-Cell-Specific c-*fes* Promoter Is Regulated by Sp1, PU.1, and a Novel Transcription Factor

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The protein product of the c-*fps/fes* (c-*fes*) proto-oncogene has been implicated in the normal development of myeloid cells (macrophages and neutrophils). mRNA for c-*fes* has been detected exclusively in myeloid cells and vascular endothelial cells in adult mammals. Although a 13-kilobase-pair (kb) human c-*fes* transgene exhibits high levels of expression in mice, the sequences that confer myeloid-cell-specific expression of the human c-*fes* gene have not been defined. Transient-transfection experiments demonstrated that plasmids containing 446 bp of c-*fes* 5′-flanking sequences linked to a luciferase reporter gene were active exclusively in myeloid cells. No other DNA elements within the 13-kb human c-*fes* locus contained positive *cis*-acting elements, with the exception of a weakly active region within the 3′-flanking sequences. DNase I footprinting assays revealed four distinct sites that bind myeloid nuclear proteins (−408 to −386, −293 to −254, −76 to −65, and −34 to +3). However, the first two footprints resided in sequences that were largely dispensable for transient activity. Plasmids containing 151 bp of 5′-flanking sequences confer myeloid-cell-specific gene expression. Electrophoretic mobility shift analyses demonstrated that the 151-bp region contains nuclear protein binding sites for Sp1, PU.1, and/or Elf-1, and a novel factor. This unidentified factor binds immediately 3′ of the PU.1/Elf-1 site and appears to be myeloid cell specific. Mutation of the PU.1/Elf-1 site or the 3′ site (FP4-3′) within the context of the c-*fes* promoter resulted in substantially reduced activity in transient transfections. Furthermore, transient-cotransfection assays demonstrated that PU.1 (and not Elf-1) can transactivate the c-*fes* promoter in nonmyeloid cell lines. We conclude that the human c-*fes* gene contains a strong myeloid-cell-specific promoter that is regulated by Sp1, PU.1, and a novel transcription factor.

During mammalian development, specific cells within the mesoderm differentiate into pluripotent hematopoietic stem cells that form all blood cell types, including lymphocytes, megakaryocytes, erythrocytes, granulocytes, and monocytes. Proto-oncogenes have been implicated in the regulation of cellular differentiation. Several mammalian proto-oncogenes exhibit restricted patterns of expression, suggesting that their transcription is tightly controlled and that they encode proteins with lineage-specific functions (30, 33, 38). The c-*fps/fes* (herein referred to as c-*fes*) proto-oncogene encodes a 92-kDa cytoplasmic protein-tyrosine kinase that includes a Src homology 2 domain and a carboxyl-terminal catalytic domain (29). c-*fes* is distinct from members of the Src family of tyrosine kinases in that it lacks a negative regulatory tyrosine phosphorylation site in its carboxyl-terminal region, is not modified by N-terminal myristylation, and does not have a Src homology 3 domain. Previously, the c-*fes* protein ($p92^{c\text{-}fes}$) had been detected only in hematopoietic cells in adult mammals and birds, predominantly in differentiated neutrophils and macrophages and their precursors (7, 17, 19, 29, 32). However, more recent experiments have shown that c-*fes* is also expressed in vascular endothelial cells and several rapidly proliferating embryonic tissues (3, 10).

A requirement for $p92^{c\text{-}fes}$ in myeloid-cell (monocyte and neutrophil) development has been suggested by a number of experiments. Introduction of a c-*fes* genomic clone into leukemic (K562) cells induces them to undergo terminal myeloid differentiation (40). c-*fes* kinase activity increases following treatment of HL60 cells with the granulocyte inducer retinoic acid and monocyte inducers such as 1,25-dihydroxyvitamin D_3 (32, 39). Furthermore, inhibition of c-*fes* expression with antisense oligonucleotides in HL60 cells before induction with retinoic acid leads to the activation of programmed cell death (8, 20). Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 are potent hematopoietic growth factors which stimulate the proliferation and differentiation of various hematopoietic cells, including granulocyte and macrophage progenitor cells. One study reported a direct association between $p92^{c\text{-}fes}$ and the common β chain of the human interleukin-3 and GM-CSF receptors that is catalytically activated upon ligand binding (13). These observations suggest that $p92^{c\text{-}fes}$ is involved in regulating proliferation and differentiation during myelopoiesis. It may also be essential for the survival of myeloid cells during differentiation.

A 13-kilobase-pair (kb) c-*fes* human genomic construct is expressed in transgenic mice in a tissue-specific manner and at levels comparable to that of the endogenous murine gene (11). Furthermore, expression of the human transgene in mouse bone marrow is independent of the integration site and proportional to the transgene copy number. Remarkably, this 13-kb genomic fragment includes only 446 bp of 5′- and 1.4 kb of 3′-flanking sequences and must, by definition, include all *cis*-acting DNA elements required for high levels of myeloid-cell-specific expression. To locate where these *cis*-acting elements reside and to identify *trans*-acting factors which bind to them, we have studied the expression of various c-*fes* DNA elements linked to a reporter gene in transient-transfection

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assays in two myeloid cell lines. We report that the 5′-flanking 151 bp of c-*fes* contain a strong myeloid-cell-specific promoter. This promoter contains functionally important binding sites for Sp1, PU.1, and a novel transcription factor. Also, PU.1 transactivates the promoter in nonmyeloid cell lines.

MATERIALS AND METHODS

RNase protection assay. Total RNA was isolated from the following human-derived cell lines: HeLa, Jurkat, K562, U937, HL60, and THP-1. In addition, a separate flask of U937 cells was treated with phorbol 12-myristate 13-acetate (PMA) at 54 nM for 16 h prior to RNA isolation. All cell lines were passaged 1:3 24 h prior to RNA extraction to ensure that cells were healthy. The TRIzol reagent (Gibco/BRL) for RNA purification was used as specified by the manufacturer except that two rounds of extraction were performed to completely remove contaminating genomic DNA.

Antisense c-*fes* and β-actin RNA probes were generated by in vitro transcription with T7 polymerase and linearized plasmids. The c-*fes* probe corresponds to genomic DNA positions 11273 through 11645 (GenBank accession number X06292) and protects 273 bp of exon 19. The β-actin construct (Ambion) produces a 189-bp transcript and protects a 127-bp fragment. Analysis was performed as recommended by the manufacturer.

Plasmid construction. Plasmid pSVBR91 contains the 13-kb *Eco*RI human c-*fes* genomic locus and was kindly provided by A. J. M. Roebroek. Plasmid p446 contains the c-*fes* promoter (bp −446 to +71) cloned upstream of the firefly luciferase reporter pGL2-basic (Promega). The 517-bp *Eco*RI-*Kpn*I fragment from pSVBR91 was subcloned into *Sma*I–*Kpn*I-digested pGL2. A series of 5′ deletions were made by subcloning a *Bss*HII-*Kpn*I fragment (yielding p366), an *Eag*I-*Kpn*I fragment (yielding p151), and a *Sma*I-*Kpn*I fragment (yielding p51) into pGL2. The p251 and p99 constructs were generated by subcloning PCR-amplified products into pGL2-basic. Site-directed mutagenesis was performed with the following oligonucleotides: for PU.1, 5′-GGGGCCTGGGCCAACTGAAACCGCGGGAGCACCGGGCGCGGAATCAGGAACTGGCCGGGGTCCGCACCGGGCCTGAGTCGGTCCGGTAC-3′, and for FP4-3′, 5′-GGAGGAAGCGCGCCACCAGGAACTGGCC-3′. The underlined sequences indicate the nucleotides that were changed from wild-type sequences. A plasmid containing a mutation in the putative Ets site was made by first subcloning a construct harboring an Ets site mutation in 51 bp of 5′-flanking sequences and then cloning an *Eco*RI-*Sma*I partial restriction fragment upstream. Mutations in the potential AP-1 site were generated by PCRs with mutant primers. Constructs containing downstream sequences were generated by subcloning six distinct *Sac*I fragments into the pGL2 promoter plasmid that contains a simian virus 40 (SV40) promoter 5′ of the luciferase gene or four distinct *Bam*HI fragments into a 3′ site of p446. A 1.4-kb *Nsi*I-*Eco*RI fragment from the c-*fes* genomic construct which contains 3′-flanking sequences was subcloned into both the pGL2 promoter and p446. Reference plasmids for transient transfections consisted of luciferase coding sequences with the Rous sarcoma virus promoter and growth hormone (GH) with the cytomegalovirus promoter. All plasmids were verified by dideoxy-DNA sequencing.

Cell culture and transient-expression analysis. Both U937 and K562 cells were grown in RPMI medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glutamine. Cos and HeLa cells were grown in Dulbecco modified Eagle medium containing the same additives. Approximately 24 h prior to electroporation, the cells were split 1:3 with fresh medium. The following day, U937 and K562 cells were electroporated as described previously (22). Cos and HeLa cells were transfected with Lipofectin reagent (Gibco/BRL). Duplicate transfections were performed by combining the cells, 100 μg of luciferase reporter plasmid, and 2 μg of the GH reference plasmid, pcDNA-GH, in electroporation cuvettes. At the time of plating, all of the U937 cells were stimulated with 54 nM PMA to increase the transfection efficiency as reported previously (22). Cos cells were cotransfected with 16 μg of pcDNA3 expression plasmid (Invitrogen) containing PU.1 or Elf-1, 4 μg of test luciferase reporter plasmid, and 2 μg of the GH reference plasmid. After 13 h (for U937 and K562 cells) or 48 h (for Cos and HeLa cells) of culture at 37°C, cells were harvested and assayed for protein content, secreted GH, and luciferase activity. Luciferase assays were performed with equivalent amounts of protein obtained from all cell extracts. GH activity was measured on transfected-cell medium supernatants by radioimmunoassay as specified by Nichols Institute. As a control for transfection efficiency, plasmid expression was quantitated as relative light units normalized to nanograms of GH secreted. All transfections were performed at least three times to ensure reproducibility.

Nuclear extracts and EMSAs. Nuclear extracts of U937 cells were prepared as described previously (5), assayed for protein content (Bio-Rad), and used to identify protein binding sites in the 5′ region of the c-*fes* gene between positions −446 and +71 relative to the primary transcriptional start site. Because of its length, this fragment was digested with *Eag*I (which cuts at −151), thus generating two probes containing 294 and 223 bp of DNA. DNase I footprinting assays were performed essentially as described in reference 6.

Electrophoretic mobility shift assays (EMSAs) were performed by incubating 10 μg of nuclear extract (2) with 10,000 dpm of each radiolabeled probe as described previously (14). Alternatively, radiolabeled oligonucleotide probes were incubated with in vitro-translated proteins prepared with T7-coupled reticulocyte lysates (Promega). Reactions were performed for 15 min at 4°C or 30 min at room temperature.

The following oligonucleotides were utilized: c-*fes* Sp1 (−102 to −46), 5′-agctCGGGACGGTCGGGCCGGTCCCGCCCGCCCCTTCCCCTCCACAGGCCCGCCCCGGGG-3′; Sp1 consensus, 5′-gatcCCTGGCTAAAGGGGCGGGGCTTGGCCAGCC-3′; PU.1/Elf-1 binding site, PU.1 (−32 to −1), 5′-gatcAAACCGCGGGAGGAAGCGCGGAATCAGGA-3′; PU.1/FP4-3′ (−32 to +14), 5′-agcttAAACCGCGGGAGGAGGAAGCGCGGAATCAGGAACTGGCCGGGGTCC-3′; and the PU.1 consensus from the SV40 promoter (26). The mutated PU.1 site in the PU.1 oligonucleotide (−32 to −1) and the SV40 oligonucleotide are listed in Fig. 7 and 8. The sequences utilized to analyze the flanking site binding requirements were made in the context of the c-*fes* oligonucleotide PU.1/FP4-3′ (−32 to +14) and are as follows: mutation 1 (mut1), 5′-gcttAAACCGCGGGAGGAGGAAGCGCGCCACCAGGAACTGGCCGGGGTCC C-3′; mut2, 5′-agcttAAACCCGGGAGGAGGAAGCGCGCCACGGGGAACTGGCCGGGGTCC-3′; and mut3, 5′-agcttAAACCGCGGGAGGAGGAAGCGCCCTAATTAAGGCTGGCCGGGGTCC-3′. The underlined sequences represent mutated bases. The lowercase letters indicate the sequences used for subcloning purposes. The consensus AP-1 site is from the metallothionein promoter (25). The rabbit polyclonal antisera specific for human Sp1, murine PU.1, human pan-*fos*, and murine pan-*jun* were purchased from Santa Cruz Biotechnology, Inc. A second polyclonal antibody specific for full-length PU.1 was a gift of David Kabat. A monoclonal antibody specific for Elf-1 was the generous gift of Jeff Leiden. Supershift and/or blocking reactions were performed as described above for EMSAs.

RESULTS

c-*fes* is transcribed in human myeloid cell lines. In the human genome the 3′ end of *fur*, a gene encoding a transmembrane protein with receptor-like features, is located approximately 1 kb upstream of the first exon of c-*fes* (27). Of note, *fur* and c-*fes* are expressed in distinct tissues. The 13-kb human c-*fes* genomic fragment, which displays high levels of tissue-specific expression in transgenic mice, contains 446 bp of 5′-flanking sequences and 1.4 kb of 3′-flanking sequences. The c-*fes* locus includes 19 exons, one DNase I-hypersensitive site located 5′ of the first exon, and two sites located between exons 3 and 4 (Fig. 1A) (15). Previous studies of $p92^{c\text{-}fes}$ indicated that it is preferentially expressed in myeloid cells in adults. To confirm and extend these results, we analyzed the relative levels of c-*fes* expression in various human cell lines by RNase protection analyses (Fig. 1B). RNA was isolated from four myeloid cell lines (K562, U937, HL60, and THP-1), an epithelial carcinoma cell line (HeLa), and a T-cell line (Jurkat). As shown in Fig. 1B, c-*fes* mRNA was detected in U937, HL60, and THP-1 cells but was not detectable in either the T cells or epithelial cells. K562 cells were established from a patient with chronic myelogenous leukemia and exhibit both erythrocytic and early monocytic characteristics (18). Experiments involving gene transfer into K562 cells with the 13-kb c-*fes* human genomic clone resulted in high levels of c-*fes* mRNA and kinase activity and subsequent myeloid differentiation (40). We did not detect any endogenous c-*fes* mRNA in K562 cells by RNase protection assays (Fig. 1B) but could detect low levels by reverse transcription-PCR (data not shown). c-*fes* mRNA and protein levels have also been reported to increase as monocytes and granulocytes mature. Treatment of U937 cells with phorbol esters induces terminal monocytic differentiation. Therefore, we stimulated U937 cells with PMA in order to test the hypothesis that this would induce c-*fes* transcription. However, PMA treatment for 16 h decreased mRNA levels in U937 cells (Fig. 1B). The lower panel in Fig. 1B, showing hybridization to a β-actin probe, demonstrates that similar amounts of RNA were analyzed in each sample.

Functional domains within the c-*fes* gene. Transient transfections into myeloid cells by electroporation were conducted to identify *cis*-acting sites within the 13-kb c-*fes* locus. Initially, we transfected a luciferase reporter plasmid containing bases

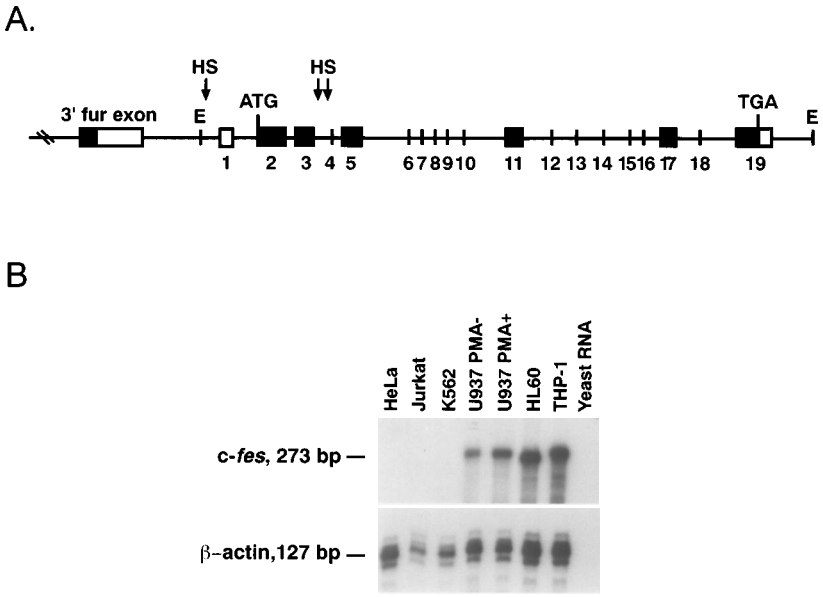


FIG. 1. (A) Schematic representation of the human c-*fes* gene. The 19 exons are indicated with coding regions (filled boxes) and noncoding sequences (open boxes). The positions of translational initiation and termination codons and DNase I-hypersensitive sites (HS) are shown. The 3′ end of *fur* is less than 1.1 kb upstream of exon 1 of c-*fes*. (B) c-*fes* mRNA production in human cell lines. Twenty-five micrograms of total RNA was hybridized to a 372-bp c-*fes* riboprobe and a 189-bp β-actin riboprobe. The positions of the 273-bp c-*fes*- and 127-bp β-actin-protected fragments are indicated. Twenty-five micrograms of yeast tRNA was included as a negative control. Autoradiograms were scanned with an Agfa Arcus Plus scanner into a Macintosh Quadra 700 computer with Adobe Photoshop software and were printed on a Rasterops CorrectPrint 300 printer.

−446 to +71 of c-*fes* into U937 cells. This plasmid (called p446) increased luciferase activity 125,000 times over that of the plasmid pGL2-basic negative control, which is expressed at very low levels in U937 cells (Fig. 2A). The level of activity obtained with the c-*fes* p446 reporter plasmid was 25% of that obtained with the RSV-luciferase positive control plasmid (Fig. 2A).

The human c-*fes* promoter contains a CpG island and no TATA element; multiple transcription start sites have been determined by primer extension analysis of U937 and HL60 mRNA (1). c-*fes* transcripts initiate at multiple sites within exon 1, and the primary site corresponds to the first nucleotide of published cDNA sequences (1). One additional cap site is located at the 5′ border of exon 2. To determine if the first intron serves as a second promoter, we linked DNA sequences from this region to luciferase coding sequences and transfected this construct into U937 cells. In contrast to the high levels of expression noted for the sequences 5′ to exon 1, sequences within the first intron contain no transcriptional activity (data not shown). To determine if other positive, *cis*-acting elements for transient c-*fes* expression exist within the 13-kb human genomic locus (in particular the third intron, where two DNase I-hypersensitive sites are located), various *Sac*I and *Bam*HI DNA fragments spanning the locus were cloned into a pGL2 plasmid containing the SV40 promoter (pGL2 promoter) or p446 (the c-*fes* promoter). The 1.4-kb 3′ region up to the *Eco*RI site indicated in Fig. 1A elevated transient expression threefold compared with either the pGL2 promoter or p446 (data not shown). Taken together, our results indicate that the strongest positive, *cis*-acting DNA elements identified by transient-transfection analyses lie within the first 446 bp 5′ of the primary mRNA cap site. Although a weak 3′ enhancer element was identified by our experiments, we focused on the 446-bp 5′-flanking region.

To further localize *cis*-acting elements in the 446-bp c-*fes* promoter, deletion analyses were performed. A schematic representation of the luciferase constructs tested by transfection is shown in Fig. 2B. DNA sequences located between nucleotides −446 and −366 were largely dispensable for transient expression, as luciferase activity in U937 cells dropped only 10% if these nucleotides were removed from the 5′ end. Plasmid p251 (containing 251 bp of 5′ sequences) retained 77% of promoter activity compared with p446. However, when the region between −251 and −151 was deleted, expression was reduced to 38%. Finally, deletion of bp −99 to −51 essentially decreased activity to less than 1% of that observed with p446 (Fig. 2B). We conclude that two important functional domains of the c-*fes* promoter reside within nucleotides extending from −251 to −151 and from −99 to −51 upstream of the primary site of transcription initiation.

The c-*fes* genomic locus contains a myeloid-cell-specific promoter. The 13-kb human c-*fes* transgene is expressed in a tissue-specific manner in transgenic mice. To determine if the 446-bp c-*fes* 5′ promoter was also myeloid cell specific, the luciferase reporter plasmid, p446, was transiently transfected into myeloid and nonmyeloid cells. As shown in Fig. 3A, The promoterless plasmid pGL2 produced low levels of luciferase activity in all four cell lines tested. p446 was expressed at high levels in two myeloid cell lines (K562 and U937) and was at least 100 times more active than in the nonmyeloid cells (Cos and HeLa). Furthermore, the 446-bp 5′-flanking region elevated luciferase activity 186-fold over pGL2 in K562 cells and 115,000-fold over pGL2 in U937 cells. These results differ significantly from the 3-fold stimulation observed in Cos cells (p446 versus pGL2) and the 1.3-fold stimulation seen in HeLa cells (Fig. 3A). In addition, p446 was not active in the Jurkat T-cell line (data not shown). It is important to note that reference GH levels for K562, Cos, and HeLa cells were identical and that U937 cells gave values two- to threefold lower. These results demonstrate that the 5′ sequences upstream of the c-*fes* transcription initiation site contain a promoter that is active in a myeloid-cell-specific fashion. Of note, we predicted that the

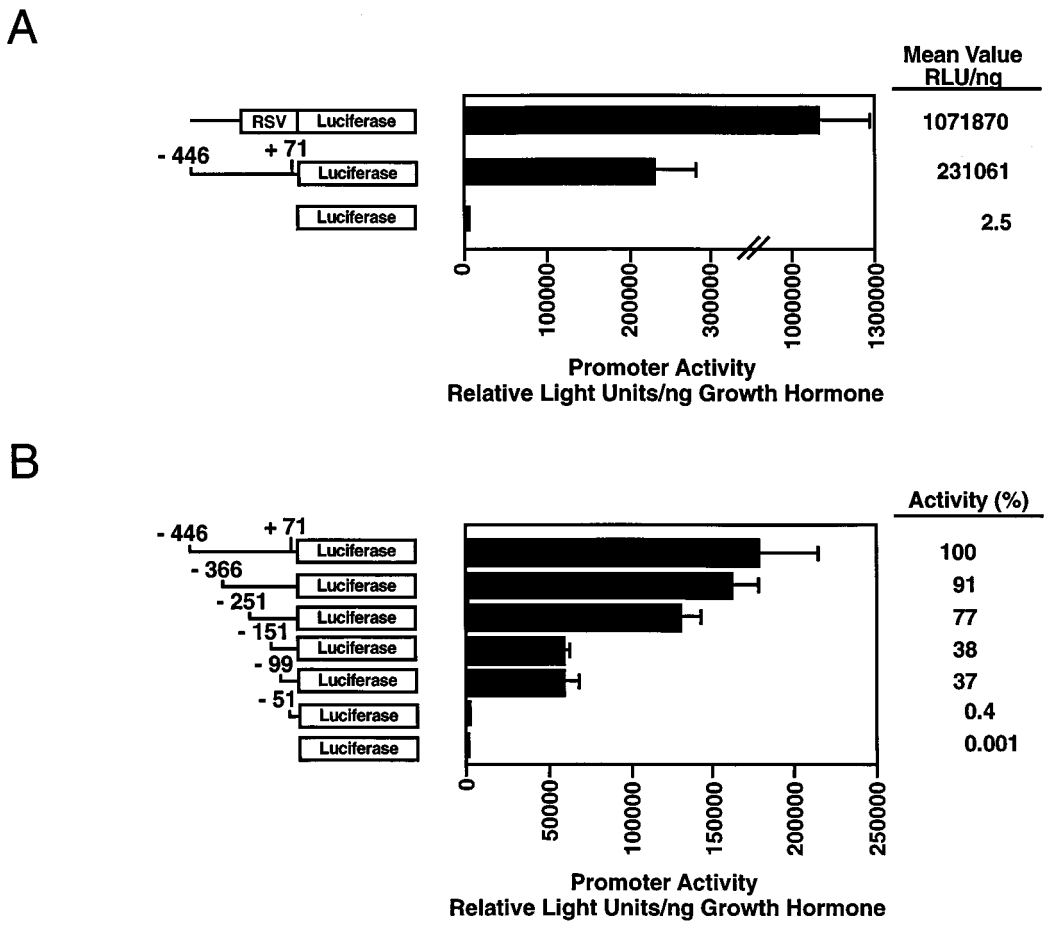


FIG. 2. (A) The 5′-flanking region of c-*fes* includes a promoter that is active in myeloid cells. One hundred micrograms of each of the three plasmids shown was transfected into U937 cells with 2 μg of pCMV-GH reference plasmid. To normalize for transfection efficiency, data are expressed as relative light units of luciferase activity per nanogram of GH secreted. Constructs are shown schematically at the left. (B) Functional analysis of the 446-bp c-*fes* promoter by 5′ deletions in U937 cells. One hundred micrograms of each of the c-*fes* luciferase reporter plasmids was electroporated with 2 μg of pCMV-GH. Each construct was electroporated 3 to 10 times with different preparations of plasmid DNA, and the data are average values of these experiments. RSV, Rous sarcoma virus.

c-*fes* promoter would be active in K562 cells even though they express low levels of the endogenous gene because the 13-kb c-*fes* transgene is active when introduced into this cell line. As stated previously, stable K562 transformants containing the transgene synthesize high levels of c-*fes* mRNA and protein and exhibit high levels of kinase activity (40).

As shown in the previous section, a plasmid containing 151 bp of 5′ c-*fes* sequences linked to luciferase was less active in U937 cells than one containing 446 bp but still resulted in 40,000-fold-higher activity than pGL2. To learn if the 151-bp region is regulated in a myeloid-cell-specific pattern, p151 was transiently transfected into U937, K562, Cos, and 3T3 cells. As shown in Fig. 3B, p151 generated luciferase activity at levels 20- to 30-fold higher in K562 and U937 cells than in 3T3 and Cos cells and thereby retained tissue-specific expression.

Sp1 interacts with the c-*fes* promoter. To identify nuclear protein binding sites within the 446-bp c-*fes* promoter, DNase I footprinting analyses were performed with nuclear extracts prepared from U937 cells. Four nuclear protein binding sites were identified in these experiments, and the data are summarized in Fig. 4. The first footprint (FP1) extends from bp −408 to −386 and does not contain a previously described consensus motif. The second nuclear protein binding site (FP2) extends from bp −293 to −254 and includes a consensus nuclear protein binding site for the transcription factor Sp1 (−293 to −285). Interestingly, neither of these two nuclear protein binding sites was absolutely required for the in vitro activity of the c-*fes* promoter (i.e., p251 retained 77% of full promoter activity) (Fig. 2B). The third footprint (FP3) includes nucleotides −76 to −65 and contains one DNase I-hypersensitive site. This region, which is footprinted weakly compared with the other footprints, contains three potential nuclear protein binding sites for the ubiquitous transcription factor Sp1. Sp1 sites have been identified in several other transcriptional regulatory elements that are present in myeloid-cell-specific genes, including CD11b (21). The fourth nuclear protein binding site (FP4) extends from −34 to +1 on the antisense strand and from −34 to +3 on the sense strand and is also surrounded by several hypersensitive sites. This region contains a consensus Ets family member binding site (−21 to −11) and a nonconsensus AP-1 binding site (−10 to −3). Comparison of this sequence with other previously described Ets binding sites suggests that this motif can bind to either or both of the transcription factors Elf-1 and PU.1 but not to Ets-1 (35, 36). In addition, as is the case for multiple other Ets binding sites (9, 34, 37), this region is immediately adjacent to a nonconsensus AP-1 family member nuclear protein binding site (5′-TGATTCC-3′, nucleotides −10 to −3 on the opposite strand).

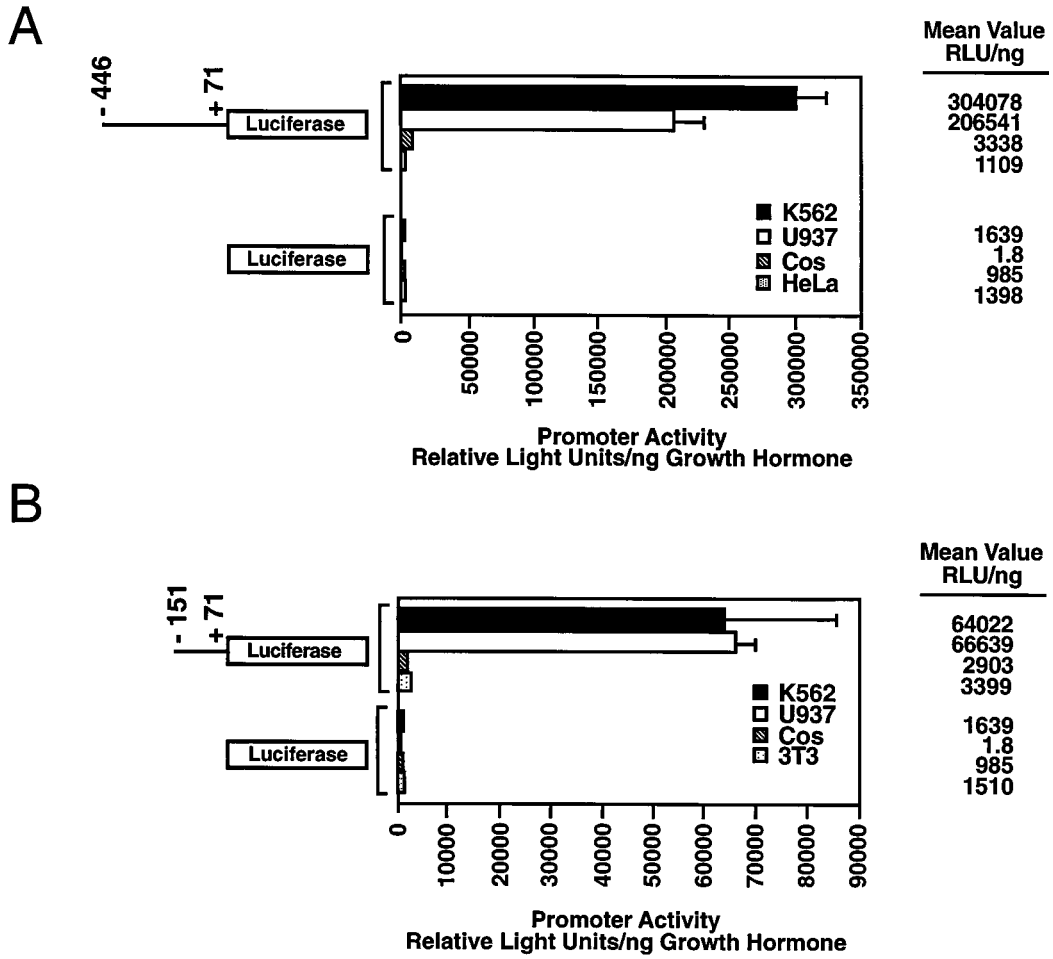


FIG. 3. (A) Functional analysis of the 446-bp 5′ promoter of the human c-*fes* gene in myeloid and nonmyeloid cell lines. K562, U937, Cos, and HeLa cells were transfected with a luciferase construct under the control of the c-*fes* promoter. K562 and U937 cells were transfected for 13 h and Cos and HeLa cells were transfected for 48 h. Each construct was transfected 3 to 10 times with different preparations of plasmid DNA, and the data are averages of these experiments. The reference plasmid, pCMV-GH, produced similar levels of GH in all cell lines transfected. (B) Functional analysis of the 151-bp 5′ region in K562, U937, Cos, and 3T3 cells.

As shown by computer search analyses, there are three putative Sp1 binding sites in the core c-*fes* promoter. To determine if Sp1 interacts with the promoter, EMSAs were conducted with an oligonucleotide probe that includes these three putative Sp1 binding sites and extends from bp −93 to −46 (Fig. 5). The first three lanes in Fig. 5A exhibit results obtained with nuclear extracts prepared from HeLa, U937, and K562 cells respectively. As expected, all three cell lines contained DNA binding activities for the ubiquitous Sp1 protein when incubated with this oligonucleotide probe. To test the specificity of binding, the assays that are shown in lanes 4 through 6 of Fig. 5 also contained various cold competitor DNAs. A 100-fold molar excess of the identical c-*fes* oligonucleotide effectively inhibited DNA binding (Fig. 5, lane 4). An unrelated oligonucleotide that includes a consensus Sp1 site (see Materials and Methods) also competed for this DNA-protein complex (Fig. 5, lane 5). However, another unrelated oligonucleotide (PU.1) corresponding to 32 bp of the c-*fes* promoter (−32 to −1) that includes a PU.1/Elf-1 binding site did not compete for this binding activity (Fig. 5, lane 6). Addition of an antibody against Sp1 caused a supershift of the DNA-protein complex in K562 cells (Fig. 5B, lane 2). These results strongly suggest that transcription factor Sp1 binds in vitro to at least

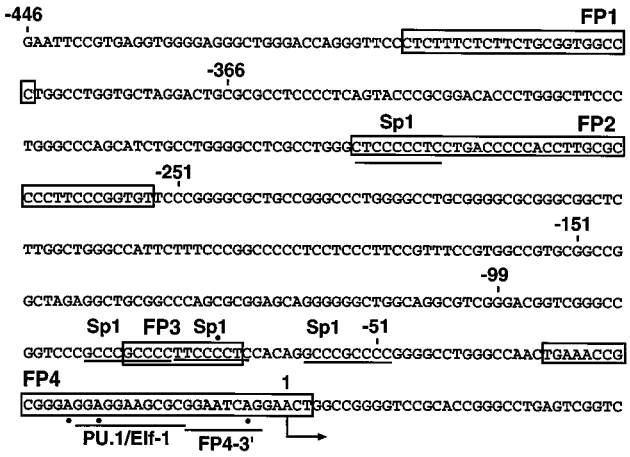


FIG. 4. Nucleotide sequence of the c-*fes* 446-bp 5′ region. Nuclear protein binding sites identified with U937 nuclear extracts and footprint analyses are boxed. Filled circles above the sequence indicate hypersensitive sites on the sense strand, and those below represent hypersensitive sites on the antisense strand. Consensus sites for Sp1 and PU.1/Elf-1 are underlined. A nonconsensus AP-1 site, 5′-TGATTCC-3′, is on the opposite strand. FP4-3′ is underlined. The primary mRNA cap site is indicated by an arrow.

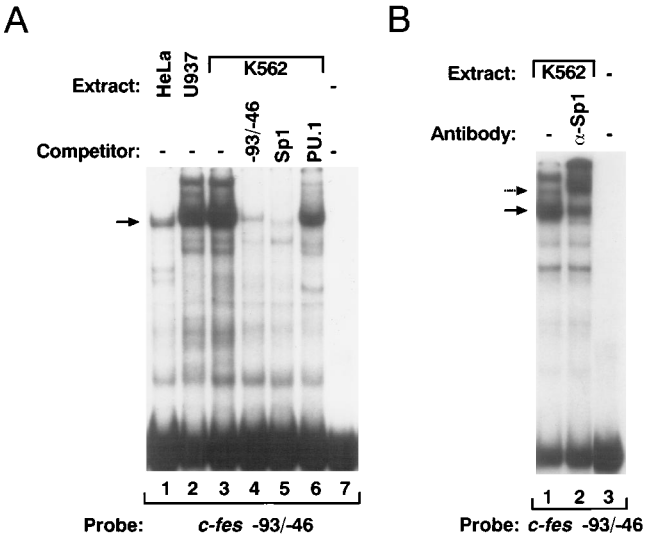


FIG. 5. EMSA by using radiolabeled c-*fes* oligonucleotide probes extending from −93 to −46 with nuclear protein extracts prepared from HeLa, U937, and K562 human cell lines. (A) EMSA with the indicated competing oligonucleotides in lanes 4 through 6. Where indicated, 100 ng (a 100-fold molar excess) of an unlabeled competitor oligonucleotide was added to the binding reactions. (B) EMSA with the addition of antibody that specifically reacts with the human Sp1 protein. Arrows, Sp1 complex; dashed arrow, Sp1 supershifted complex.

one of the three consensus Sp1 sites in this portion of the c-*fes* promoter.

The PU.1/Elf-1 and FP4-3′ sites are required for c-*fes* promoter activity in myeloid cells. As shown in Fig. 2B, a construct containing 51 bp of 5′ c-*fes* sequences was inactive in transient assays. However, a footprinted region (FP4, from −34 to +3) was detected by DNase I protection analyses (Fig. 4). We therefore tested specific mutations in this footprinted region in the context of the entire 446-bp promoter by transient transfections. Figure 6A shows that promoter activity in U937 cells is sharply reduced (by approximately 90%) if the PU.1/Elf-1 DNA binding site is changed from 5′-GAGGAGGAAGCG C-3′ to 5′-GAGCACCGGGCGC-3′, a mutation that renders it incapable of binding PU.1 or Elf-1 (see Fig. 7A). In addition, a mutation in the flanking site FP4-3′ (which does not affect PU.1 DNA binding activity) from 5′-TGATTCC-3′ to 5′-TGG TGGC-3′ resulted in an 80% decrease in expression compared with the wild-type construct (Fig. 6B). Taken together, these data strongly suggest that important regulators for c-*fes* expression include Sp1 and PU.1 or Elf-1. In addition, either AP-1 or another DNA-binding protein(s) binding to FP4-3′ also plays a role in the expression of c-*fes* in myeloid cells.

PU.1 and Elf-1 bind to the Ets site in the c-*fes* promoter, but AP-1 family members do not bind to FP4-3′. DNase I footprinting experiments demonstrated the existence of a nuclear protein binding site within the c-*fes* promoter that could potentially bind both Ets and AP-1 family members. c-*fes* lacks a functional TATA box and has the sequence 5′-GAGGAGG

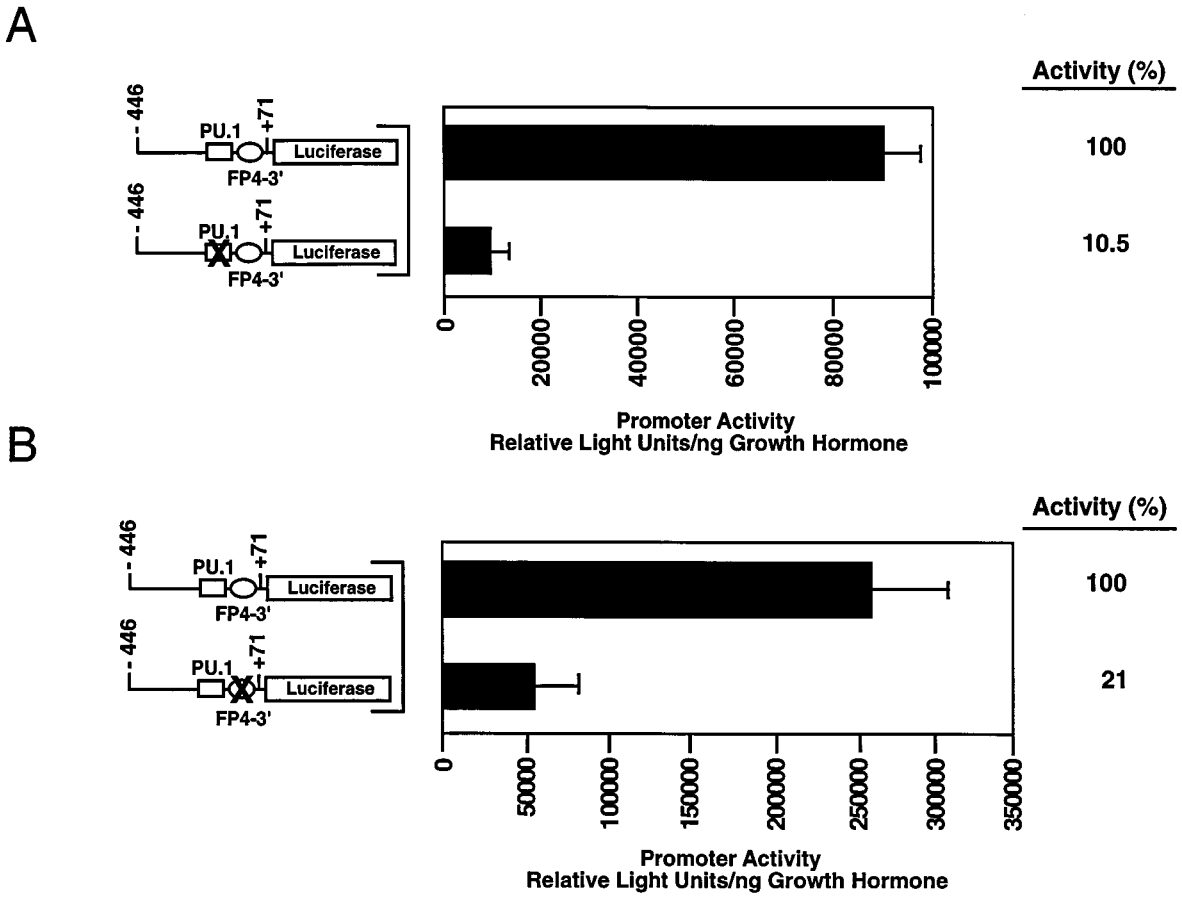


FIG. 6. Functional analysis of the PU.1/Elf-1 and FP4-3′ sites in U937 cells. (A) The 446-bp promoter with a mutation in the PU.1/Elf-1 site was electroporated into U937 cells. (B) The 446-bp promoter with a mutation in the adjacent FP4-3′ site was electroporated into U937 cells.

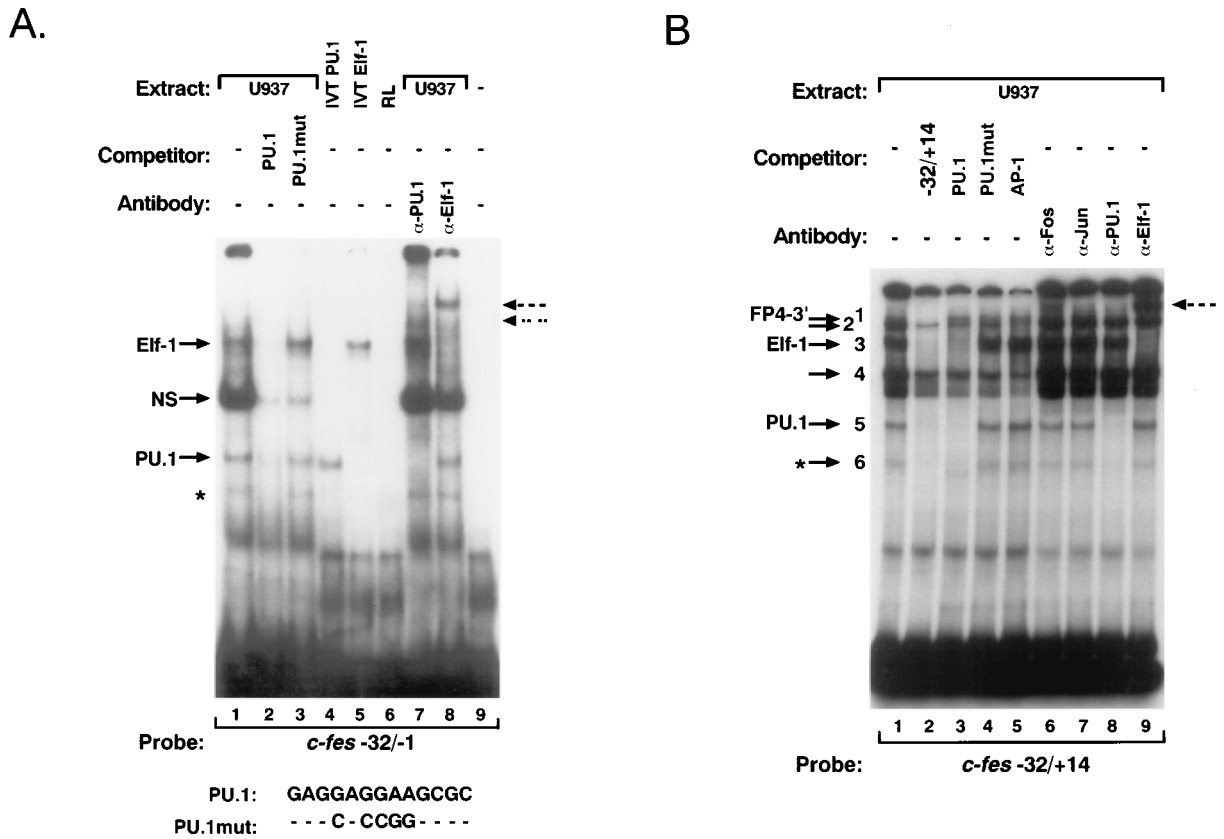


FIG. 7. Investigation of protein binding to the PU.1/Elf-1 and FP4-3′ sites. (A) EMSA by using radiolabeled c-*fes* probe (−32 to −1) with U937 nuclear protein extracts and in vitro-translated PU.1 and Elf-1 proteins. Lanes 2 and 3 include 100 ng of the indicated unlabeled competitor oligonucleotide. Lane 6 contains the same amount of unprogrammed reticulocyte lysate as that programmed with PU.1 pcDNA (lane 4) and Elf-1 pcDNA (lane 5). Lanes 7 and 8 include the indicated antibodies to PU.1 and Elf-1. The mutated nucleotides in the mutant PU.1 oligonucleotide are shown below the autoradiogram. NS, nonspecific nuclear protein complex; IVT, in vitro translated; RL, reticulocyte lysate. Solid arrows, DNA-protein complexes; dashed arrow, Elf-1 supershift; dotted arrow, PU.1 supershift; asterisk, fastest-migrating complex; α, anti. (B) AP-1 family members failed to bind to a c-*fes* probe extending from −32 to +14 (PU.1/FP4-3′). Lanes 2 through 5 contain 100 ng of the indicated cold competitor oligonucleotide. Lanes 6 through 9 contain the indicated antibodies. Numbers at left indicate bands. Dashed arrow, Elf-1 supershift.

AAGC-3′ at position −21 upstream of the transcription start site. Myeloid cells express the Ets family members Ets-1, Elf-1, and PU.1 (2b, 16). Published DNA binding site specificities indicate that the c-*fes* promoter contains a consensus Elf-1 and/or PU.1 site: Ets-1 selectively binds 5′-CAGAGGATGT G-3′, while both Elf-1 and PU.1 bind the 5′-GAGGAGGAA GC-3′ sequence (35, 36). To determine if myeloid-cell nuclear extracts contain Ets binding activities that recognize this region of the c-*fes* promoter, EMSAs were performed. As shown in Fig. 7A, a radiolabeled oligonucleotide corresponding to bp −32 to −1 forms multiple nuclear protein complexes with extracts prepared from U937 cells. Three complexes evince specific interactions with the oligonucleotide probe because a 100-fold molar excess of unlabeled oligonucleotide competes for these DNA binding activities (Fig. 7A, lanes 1 and 2). The fourth complex, is thought to be nonspecific, as an oligonucleotide with a mutation in the Ets site also competed for binding (Fig. 7A, lane 3), as did a variety of oligonucleotides with dissimilar sequences (data not shown). We have determined that the most slowly migrating complex contains Elf-1, as it comigrates with in vitro-translated Elf-1 protein (Fig. 7A, lane 5) and is supershifted with a monoclonal antibody that specifically recognizes Elf-1 and not other Ets family members (Fig. 7A, lane 8). In addition, a more rapidly migrating complex represents PU.1 binding to the Ets site, as it comigrates with in vitro-translated PU.1 (Fig. 7A, lane 4) and is blocked by the addition of anti-PU.1 antibody (Fig. 7A, lane 7). Both antibodies used in these experiments were tested with pure Elf-1 and PU.1 proteins (see Fig. 8; also, data not shown). The fastest-migrating complex may represent a proteolytic product of PU.1 that retains DNA binding activity. However, it is not possible to unequivocally identify this protein, as it is not recognized by the antibody to PU.1.

To determine if AP-1 family members bind to the nonconsensus site at −10 to −3, a longer oligonucleotide which extended from −32 to +14 (−32/+14) and included FP4-3′ was utilized. By using this oligonucleotide and unstimulated U937 nuclear extracts, six nuclear protein complexes were detected (Fig. 7B). The upper two bands in Fig. 7B migrate very closely together and are resolved best in lane 3. Band three corresponds to an Elf-1 complex because it can be supershifted with an Elf-1-specific antibody (Fig. 7B, lane 9). Similarly, bands five and six are identified as PU.1 by their ablation with the anti-PU.1 antibody, which now detects both intact and truncated PU.1 (Fig. 7B, lane 8). To identify the proteolytic product of PU.1, binding reactions were modified and tested with antibodies that recognize the amino terminus (Santa Cruz) and full-length protein (from David Kabat, Oregon Health Sciences University). Bands five and six are also inhibited by competition with the PU.1 oligonucleotide (−32 to −1) (Fig. 7B, lane 3). Therefore, in an investigation of the nonconsensus AP-1 site, bands one, two, and four were considered. Band two

was not inhibited by cold oligonucleotide −32/+14 and was therefore thought to be nonspecific (Fig. 7B, lane 2). Neither band one nor band four was supershifted or reduced by anti-pan-*fos* or anti-pan-*jun* antibodies (Fig. 7B, lanes 6 and 7). However, both of these antibodies affected specific AP-1-shifted bands detected in U937 cells treated with PMA by using a consensus metallothionein AP-1 probe (data not shown). Bands one and four were also not inhibited upon addition of a cold competitor consensus AP-1 oligonucleotide from the metallothionein promoter (Fig. 7B, lane 5). We concluded that the diffuse band four corresponds to the nonspecific band indicated in Fig. 7A and that band one corresponds to a protein binding FP4-3′ (see below). We also concluded that AP-1 does not regulate c-*fes*.

PU.1 binds to the c-*fes* promoter at two sites, −21 to −14 and −8 to +3. A recent report (26) claims that in vitro-translated PU.1 binds to the sequence 5′-AATCAGGAACT-3′ located at nucleotides −8 to +3 of the c-*fes* promoter. The oligonucleotide −32/+14 was used in EMSAs with in vitro-translated PU.1. As shown in Fig. 8, PU.1 binds to the PU.1/FP4-3′ (−32/+14) oligonucleotide (lane 1) and is specifically inhibited by excess cold competitor (lane 2). Importantly, the PU.1 oligonucleotide (−32 to −1) also competes for binding (Fig. 8, lane 3), while a mutation at the Ets site at −21 no longer competes (Fig. 8, lane 4). This demonstrates that the −32/−1 oligonucleotide does not include the complete PU.1 site from −8 to +3. Mutations 1, 2, and 3 of the flanking site FP4-3′ (−8 to +3) competed efficiently in our assays (Fig. 8, lanes 8 to 10), showing that the −21 site is intact. EMSAs with the −11/+5 oligonucleotide also demonstrated binding of PU.1 in U937 nuclear extracts (see Fig. 10A, lane 1). Because both the −32/−1 oligonucleotide (Fig. 7A) and the −11/+5 oligonucleotide (Fig. 10A) bind efficiently, we conclude that PU.1 binds to the Ets site at −21 and to the site at −8. The −32/+14 oligonucleotide harbors two binding sites for PU.1 in addition to a site for the FP4-3′ activity.

Transactivation of the myeloid-cell-specific c-*fes* promoter by PU.1. Given the specific binding of both Elf-1 and PU.1 to the c-*fes* promoter, we performed a series of transient-cotransfection experiments with both of these proteins in the nonmyeloid Cos cell line, which lacks endogenous Elf-1 or PU.1 activity. The promoterless luciferase expression vector pGL2, a minimal c-*fes* promoter construct (p51), and a c-*fes* promoter construct containing a mutation in the Ets site (p51m) were introduced into Cos cells with expression plasmids that produce high levels of Elf-1 or PU.1 driven by the cytomegalovirus promoter. As shown in Fig. 9, cotransfection with PU.1 resulted in a reproducible fivefold stimulation of the c-*fes* promoter, and this stimulation was dependent on having the −21 Ets binding site intact. Similar results were obtained in experiments carried out with 3T3 cells (data not shown). Importantly, PU.1 transactivated constructs containing the full promoter (data not shown). In contrast, Elf-1 failed to transactivate the c-*fes* promoter in either cell line. On the basis of these results, we conclude that PU.1 is the most likely member of the Ets family of DNA-binding proteins to regulate c-*fes* gene expression. Furthermore, when the Ets site at position −21 is mutated, PU.1 fails to transactivate the c-*fes* promoter even though the PU.1 binding site at −8 is intact.

A novel factor binds to the FP4-3′ site in the c-*fes* promoter. Our results clearly demonstrate that PU.1 binds specifically to the Ets site at nucleotide −21 of the c-*fes* promoter and that transactivation depends on the integrity of the −21 site. We performed EMSAs to determine what factor(s) binds to FP4-3′ (−8 to +3), having determined that AP-1 does not bind to FP4-3′ (Fig. 7B). These EMSAs utilized a shorter oligonucleotide probe (−11 to +5) in order to isolate the FP4-3′ binding interaction. Figure 10A shows the results of EMSAs performed to determine where the FP4-3′ complex binds within the oligonucleotide −11/+5. Lanes 1 through 5 of Fig. 10A demonstrate that the most slowly migrating complex corresponds to the FP4-3′ binding activity. This band is inhibited by the wild-type oligonucleotide (Fig. 10A, lane 2) but not by any of the three mutations (Fig. 10A, lanes 3 through 5). Importantly, a mutation in the FP4-3′ site that affects the sequence 5′-GAATCAGGAACT-3′ (mut1) interrupts binding and decreases c-*fes* expression in transient-transfection assays (Fig. 6B). This mutation still allows PU.1 to bind to the −8 site. Lanes 6, 7, and 10 of Fig. 10A confirm that this FP4-3′–protein complex does not contain PU.1. Neither a consensus PU.1 oligonucleotide from SV40 nor a PU.1 antibody disrupts the FP4-3′ complex. However, the SV40 PU.1 oligonucleotide specifically inhibits the PU.1 complex (the fastest two bands in Fig. 10A, lanes 6 and 7). Furthermore, FP4-3′ is not affected by Sp1 or AP-1 oligonucleotides or by antibodies to PU.1, Myb, Elf-1, Fos, or Jun (Fig. 10A, lanes 8 through 14).

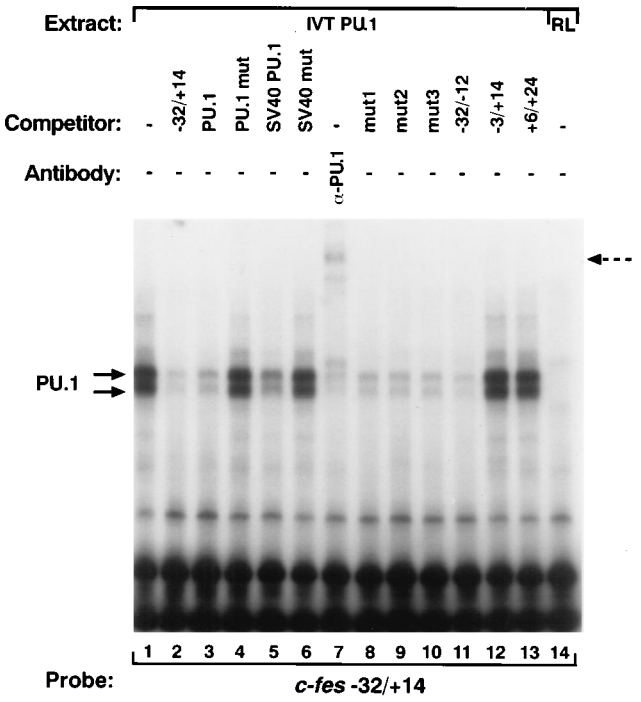


FIG. 8. EMSA with in vitro-translated PU.1 and the c-*fes* −32/+14 oligonucleotide as a probe. Lanes 2 through 6 and 8 through 13 contain the indicated cold competitors. The oligonucleotide PU.1 is from bp −32 to −1 of the c-*fes* promoter. The oligonucleotide PU.1 mut is the same as in Fig. 7A. Other mutations are given below the autoradiogram. The numbers in lanes 2 and 11 through 13 represent the first and last bases of the c-*fes* promoter, which correspond to the oligonucleotides used as cold competitors. IVT, in vitro translated; RL, reticulocyte lysate. Solid arrows, DNA-protein complexes; dashed arrow, supershifted complex; α, anti.

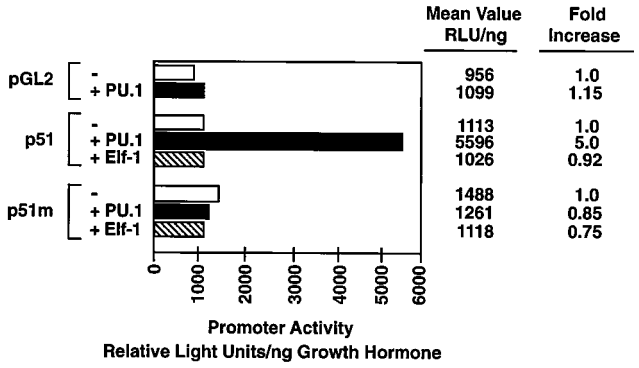
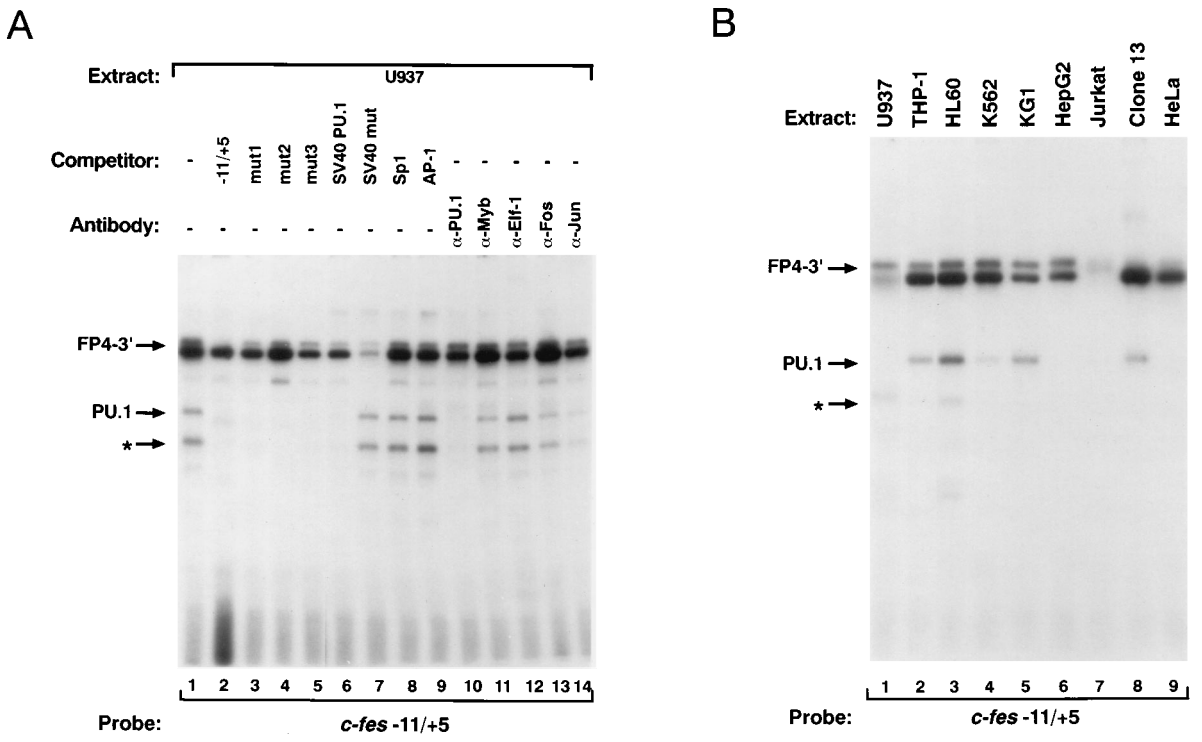


FIG. 9. Cotransfection of the c-*fes* promoter into Cos cells with pcDNA-PU.1 and pcDNA-Elf-1. Four micrograms of each of the indicated c-*fes* plasmids was lipofected into Cos cells along with 16 μg of either pcDNA-PU.1 or pcDNA-Elf-1. p51m is a c-*fes* plasmid that is identical to p51 except that the PU.1 binding site has been mutated to the site shown in Fig. 6. Control experiments included 16 μg of empty pcDNA construct. In addition, 2 μg of pCMV-GH was included to normalize lipofection efficiencies.

To determine the tissue distribution of the FP4-3′-binding factor, EMSAs were performed with nuclear extracts from myeloid (U937, THP-1, HL60, K562, and KG1) and nonmyeloid (HepG2, Jurkat, Clone 13, and HeLa) cell lines (Fig. 10B). The FP4-3′-specific complex was present only in the nuclear extracts from the myeloid cell line, with the exception of HepG2 liver cells, which also contain the activity. The slowly migrating complex was not detected in Jurkat T-cell, Clone 13 B-cell, or HeLa epithelial-cell nuclear extracts. We conclude that the FP4-3′ complex is largely restricted to myeloid cells.

DISCUSSION

The c-*fes* tyrosine kinase appears to be highly restricted in its pattern of expression in adult mammals. Although it is expressed in other tissues in murine and human embryos, after birth it is detected only in cells of the myeloid lineage (monocytes and neutrophils and their precursors) and in vascular endothelial cells. Several lines of evidence strongly implicate the c-*fes* protein as being crucial for monocytic and neutrophilic development: increased levels of kinase activity correlate with the maturation of monocytes and neutrophils (31, 38), and inhibition of c-*fes* mRNA translation into protein leads to apoptotic death during neutrophilic differentiation (8, 20). Re-




```
c-fes -11/+5:                CGGAATCAGGAACTGG
PU.1/FP4-3':  GAGGAGGAAGCGCGGAATCAGGAACTGG
mut1:         ---------------CC-C----------
mut2:         ---------------CC-CGG--------
mut3:         --------------CCT-ATTAAGG----

SV40 PU.1:    AAAGAGGAACTT
SV40 mut:     ---C-CCGG---
```

FIG. 10. Location of the FP4-3′ site-protein complex on oligonucleotide −11/+5. (A) EMSA with U937 nuclear extracts and the c-*fes* −11/+5 probe. Lanes 2 through 9 contain 100 ng of the indicated cold competitor oligonucleotide. Lanes 10 through 14 contain the indicated antibodies. Mutations 1, 2, and 3 were tested in competing oligonucleotides including bp −32 to +14. (B) The c-*fes* −11/+5 probe was used with the various indicated nuclear extracts. Solid arrows, DNA-protein complexes; asterisk, proteolyzed PU.1; α, anti-.

markably, important *cis*-acting DNA elements that ensure high levels of c-*fes* transcription in myeloid cells must reside within a relatively short fragment of DNA: a 13-kb human transgene is expressed in the appropriate tissues at very high levels. The reasons for our interest in the c-*fes* gene are many. We want to learn which proteins are responsible for tightly controlling c-*fes* mRNA production. If c-*fes* kinase levels are critical for myeloid development, it stands to reason that the factors controlling its transcription will also be important in development. Also, a potent promoter for the myeloid lineage will be a useful tool for the generation of transgenic mice that inappropriately express gene products in these lineages. Given the extremely high levels of expression reported for a 13-kb human genomic fragment upon introduction into transgenic mice, we reasoned that it should be relatively important to identify *cis*-acting sites within this short region of genomic DNA.

We have demonstrated that promoter-proximal sequences from −151 to +71 are sufficient to direct myeloid-cell-specific expression. The elements that appear to be the most important for activity during electroporation into U937 cells are located at nucleotides −99 to −51 and −34 to +3. We have shown that the −99 region selectively binds the transcription factor Sp1. The footprinted site at −34 includes a consensus site for Ets family members PU.1 and/or Elf-1 as opposed to Ets-1. Although both PU.1 and Elf-1 recognize this site in in vitro binding reactions, PU.1 alone can transactivate the c-*fes* promoter in nonmyeloid cell lines (Cos and 3T3 cells), suggesting that PU.1 is the critical regulator of the c-*fes* promoter. Furthermore, macrophages that lack Elf-1 (because of gene targeting) still express normal levels of c-*fes* (2a). We have previously shown that mice deficient in PU.1 do not produce mature macrophages and neutrophils (31). The blockage of macrophage and neutrophil development is coincident with c-*fes* expression in these lineages. We have isolated early myeloid cells lacking PU.1 and demonstrated a deficiency in c-*fes* expression (21a). Therefore, our genetic data are consistent with the transactivation results showing that PU.1 is the Ets family member that regulates c-*fes.*

PU.1 has been proposed as a regulator of other myeloid-cell-specific genes, including CD11b (23), the M-CSF receptor (41), and the macrophage scavenger receptor (37). Interestingly, as is the case for CD11b and the M-CSF receptor, PU.1 binds to a site just 5′ of the primary transcriptional start site in the c-*fes* promoter, which also lacks a consensus TATA box. Given the finding that PU.1 binds to TATA-binding protein in vitro (12), it is tempting to speculate that PU.1 may function to recruit TFIID and then the other components of the basal transcriptional machinery to all three of these promoters.

The footprinted promoter-proximal region from −34 to +3 also includes a weak consensus site for AP-1 family members that is directly adjacent to the Ets site. Many Ets family transcription factors interact with another protein when binding to DNA (4, 9, 34). Combinatorial interactions between Ets-2 and AP-1 for the scavenger receptor promoter (37) and between Elf-1 and AP-1 for the GM-CSF promoter (34) have been described. PU.1 has been demonstrated to form a complex with the B-cell factor NF-EM5 to activate the immunoglobulin κ and λ 3′ enhancers (24). However, EMSAs performed with the PU.1 site in the c-*fes* promoter failed to detect PU.1 in a stable complex with another protein. A distinct assay for protein-DNA interactions (i.e., DNase I footprinting) clearly showed that both the PU.1 and FP4-3′ sites bound nuclear proteins in U937 cells. Furthermore, both sites are functionally important for c-*fes* promoter activity in U937 cells. It seems possible that a PU.1/FP4-3′–protein interaction, while important to the regulation of c-*fes* expression, may not be sufficiently stable in a gel assay for detection. It is clear that the FP4-3′ site (5′-GAATCAGGAACT-3′) does not bind either of the other myeloid transcription factors c-myb (5′-CAAC GG-3′) and CEBP/α (5′-GTGGAAAG-3′), as their consensus sites are distinct from FP4-3′ (21) and FP4-3′ does not bind antibodies specific for myb.

While this work was in progress, Ray-Gallet et al. reported that PU.1 binds to the c-*fes* promoter (26). However, their analyses suggested that PU.1 binds to a site located at −8 to +3 of the promoter. The oligonucleotide used for EMSAs in that report did not include the PU.1 site that we have identified and would therefore fail to detect PU.1 binding at bp −21 of the c-*fes* promoter. The −8 site overlaps the FP4-3′ site, which is located at −9 to +3 and for which we have demonstrated specific binding of a novel factor. Through our mutational analysis, we have identified a slowly migrating complex corresponding to a protein-DNA interaction at FP4-3′ which is critical for promoter function (Fig. 10B). This band is not supershifted or reduced by anti-PU.1 or by anti-Elf-1. We conclude that FP4 includes two binding sites for PU.1 in addition to FP4-3′. It is interesting that the Sp1, PU.1, and FP4-3′ sites have been conserved in the chicken, feline, and human c-*fes* promoters. Finally, the FP4-3′ complex appears to contain a DNA binding activity restricted in its expression to myeloid cells. It will be of interest to identify the factor(s) binding to FP4-3′ that regulates c-*fes* in myeloid cells. Such a factor(s) could be useful for the further analysis of myeloid-cell-specific expression.

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