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Opposing Actions of c-ets/PU.1 and c-myb Protooncogene Products in Regulating the Macrophage-specific Promoters of the Human and Mouse Colony-stimulating Factor-1 Receptor (c-fms) Genes

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Summary

The receptor for macrophage colony stimulating factor (CSF-1), the c-fms gene product, is a key determinant in the differentiation of monocytic phagocytes. Dissection of the human and mouse c-fms proximal promoters revealed opposing roles for nuclear protooncogenes in the transcriptional regulation of this gene. On the one hand, c-ets-1, c-ets-2, and the macrophage-specific factor PU.1, but not the ets-factor PEA3, trans-activated the c-fms proximal promoter. On the other hand c-myb repressed proximal promoter activity in macrophages and blocked the action of c-ets-1 and c-ets-2. Basal c-fms promoter activity was almost undetectable in the M1 leukaemia line, which expressed high levels of c-myb, but was activated as cells differentiated in response to leukemia inhibitory factor and expressed c-fms mRNA. The repressor function of c-myb depended on the COOH-terminal domain of the protein. We propose that ets-factors are necessary for the tissue-restricted expression of c-fms and that c-myb acts to ensure correct temporal expression of c-fms during myeloid differentiation.

The CSF-1 receptor is encoded by the c-fms protooncogene and is required for the growth, differentiation, and survival of cells in the monocyte/macrophage lineage (1). The human c-fms gene is located on chromosome 5 in a tandem array with the platelet-derived growth factor receptor gene (2). c-fms is expressed in monocyte/macrophages and their committed precursors and in placental trophoblasts (1, 3). Two distinct promoters appear to be at least partly responsible for the tissue-specific expression of the human gene (3). The placental promoter is located in the 3′ region of the platelet-derived growth factor receptor adjacent to noncoding exon 1 of c-fms, whereas the monocyte-specific promoter is located in the segment of intron 1 adjacent to exon 2, which contains the start codon (3). In contrast to the tissue-restricted activity of the human promoter, the mouse proximal promoter was demonstrated to be active in a wide variety of mouse cell lines (4). The effects of sequences in intron 2 (the first intron transcribed in macrophages) and the detection of incomplete transcripts suggested that an attenuation/elongation mechanism was responsible for directing tissue-specific expression of the gene (4).

The ets family of transcription factors contain a conserved DNA binding domain in the COOH-terminal region of the protein products and recognize a common purine-rich consensus sequence (5, 6). Ets factors act at enhancer elements in combination with AP-1 family members to direct oncogene-responsive gene expression (7, 8). The ets-factors c-ets-1 and PU.1 mediate tissue-specific expression of genes in hematopoietic cell types (9–12), in combination with other transcription factors such as the immunoglobulin-enhancer binding factor NF-E2 (13). PU.1 and c-ets-2 can both trans-activate the murine c-fms promoter in nonmacrophages. In macrophages, which already express PU.1, overexpression of this factor was repressive whereas c-ets-2 was stimulatory (14). Similar studies of the human promoter identified a PU.1-responsive element around 170 bp upstream from the start codon that activated transcription in nonmacrophages and was essential for maximal expression in macrophages (15).

The c-myb gene also encodes a DNA binding protein that acts as a transcriptional activator (16–20). The c-myb product is expressed primarily in hematopoietic cells (21) and is essential for the normal differentiation. A homozygous null mutation of this gene in transgenic mice resulted in the loss of hematopoietic precursors and embryonic lethality (22).
expression of c-myb decreases during differentiation (23, 24) and exogenous expression of myb blocks the differentiation of hematopoietic cell lines (25-27). Only a few actual targets of c-myb are known, including mim-1 (28), a promyeloid-specific gene of unknown function, and the enhancers for the CD4 gene, the cdk2 gene, and the T cell receptor δ chain (29-31). In all of these cases, c-myb is a positive regulator of gene expression. Activation of the mim-1 promoter by c-myb has been reported to depend on interactions between c-myb and ets factors (32) and more recently between c-myb and CAAT/enhancer-binding factors (33, 34). Similarly, interaction between c-myb and the T cell factor CBF is critical for cell-specific activation of the δ chain enhancer (31).

In the present work, we studied the elements contributing to the tissue-specific use of the human and murine c-fms proximal promoters, focusing on the role of ets-factors and of c-myb. A combination of transient transfection assays and in vitro protein binding assays demonstrated that ets-related factors, including c-ets-1, c-ets-2, and PU.1, may contribute as positive factors to the tissue-specific potential of the c-fms promoter. The c-myb product also recognized sequences in the c-fms proximal promoter, but this interaction caused a cell-specific negative regulation of the promoter. The COOH-terminal regulatory domain of c-myb was required for this negative regulation of the c-fms proximal promoter. c-fms is identified as a potential target for the effects of c-myb on myeloid differentiation.

Materials and Methods

Cell Culture. NIH 3T3 fibroblasts and COS-7 cells were cultured in Dulbecco's modified Eagles medium (high glucose formulation) supplemented with 10% FCS, 9.5 mM L-glutamine, and 100 µg/ml meclozilin, and grown in 5% CO2 at 37°C. The murine macrophage cell lines RAW264 (35), the myeloid leukemia M1, and the erythroleukemia cell line K562 (all obtained from American Type Culture Collection, Rockville, MD) were grown in RPMI medium containing 9.5 mM L-glutamine and 5 or 10% FCS (the former for K562).

Reporter and Expression Plasmids. The CSFR-5000 plasmid was constructed by placing the proximal human CSF-1R promoter fragment that contained 5 kb of sequences upstream of the initiation codon 5' to a luciferase reporter gene (8, 20). The CSFR-900 reporter contained a proximal promoter fragment truncated by HindIII digestion, whereas the CSFR-430 and CSFR-367 plasmids were constructed using PCR fragments corresponding to -430 to +1, or -367 to +1, respectively, of the proximal CSF-1R promoter. The primers used for PCR were 5'-GGGATCCGGATCCGTGAGAGAAGAGGATCAAAACAA -3' and 5'-GGGGATCCGGATCCGTGAGAGAAGAGGATCAAAACAA -3' for 5' ends, respectively, and 5'-GGAGATCTGAGAACTCTGGTTTGGACA-3' for the 3' end. After cloning, these PCR fragments were sequenced to ensure that mutations were not introduced by the PCR amplification. All of these promoter fragments were placed in luciferase reporters in either BlueScript (Promega Biotech, Madison, WI), or PBR322 vectors. The results presented here were obtained with the Bluescript vectors, but data with both vectors were identical. This result renders unlikely the prospect that vector sequences contributed to the regulation observed. The murine c-fms promoter constructs, pGL2-3.5fms and pGL2-0.3fms, were produced as described previously (4).

The ets-related purine-rich sites located beginning at position -151 were altered in the CSFR-430 reporter by PCR site-directed mutagenesis (8) using the oligonucleotide (sense strand) 5'-GCCCAAGTATAGTAAAGGAGGAAACCAAG-3' and its complement along with the oligonucleotides that define the boundaries of the CSFR-430 construct described above.

The myb-expression vectors contained the long terminal repeat promoter of Rous sarcoma virus and an intron-polyA processing site derived from SV40 (20). The COOH-terminal truncated c-myb construct contained amino acids 1-437, thus deleting the COOH-terminal 199 amino acid residues of the full-length protein (36, 37). The expression vector for c-ets-2 and PEA3 (38) was a cytomegalovirus immediate early promoter-based vector that contained the influenza hemagglutinin epitope tag recognized by monoclonal antibody 12CA5-F (39). The SV40 early promoter c-ets-1 vector encoded the 54-kd gene product, and was a gift from Jaques Gysdael (Institut Curie, Orsay, France). SV40-based expression plasmids for murine c-ets-2 and PU.1 (ets-2 pECE and PU-pECE) were a gift from Dr. Richard Maki (La Jolla Cancer Research Foundation; La Jolla, CA).

Transfection Assays. NIH 3T3 cells were transfected either by the calcium phosphate method (8) or by electroporation (40). In some experiments a plasmid encoding the secreted alkaline phosphatase gene was included as an internal control (8). For the calcium phosphate method, 3 µg of reporter were included per experiment, whereas the amount of expression vector for the ets-related or myb-derived proteins was 0.25 µg, determined empirically as optimal. The RAW264, M1, and K562 cells were transfected by electroporation as described (40). The amount of plasmid used for the electroporation experiments is recorded in the individual figure legends. Luciferase and secreted alkaline phosphatase reporters were assayed as described (8, 20). All transfection experiments reported were performed a minimum of three times in duplicate with at least two different preparations of plasmid DNA.

COS-7 cells were transfected by the calcium phosphate method using 15 µg of expression vector per dish for c-ets-2 or c-myb. The whole cell extracts were prepared 48 h after transfection as described (41). Overexpression of the protein was confirmed by Western blotting using the monoclonal antibody 12CA5-F (data not shown).

Preparation of Nuclear Extracts and Mobility Shift Assays. Preparation of nuclear extracts from RAW 264 or NIH 3T3 cells and electrophoretic mobility shift assays (EMSA) were accomplished exactly as described earlier (8, 14). The double-strand oligonucleotides based upon the -150 human c-fms element was 5'-GCCCAAGGAGGAGGAGGAGGAAACCAAG-3' (sense strand). The sequence of the mutated oligonucleotide for the ets-binding experiments was identical to that used for site-directed mutagenesis (see above). The SV40 PU-box sequence used in EMSA was 5'-CTGAAA CGGAGACCTGGTGGTGGAGCA-3'. In the antibody supershift assays, the whole-cell extract from COS-7 cells (see above) was incubated for 10 min at room temperature with the antibody before adding the probe. Recombinant PU.1 was produced in Escherichia coli as described previously (14).

RNA Preparation and Northern Analysis. M1 cells (1-2 x 10^6/ml) were cultivated in medium with or without leukemia inhibitory factor (LIF) (ESGRO; AMRAD, Melbourne, Australia) at a concentration of 1,000 U/ml. Each day the culture was split in half by removal of cells and addition of fresh medium. The harvested cells, plus or minus LIF, were used to prepare total RNA. Extraction, Northern blotting, and cDNA hybridization were carried out as described previously (42).

1 Abbreviations used in this paper: EMSA, electrophoretic mobility shift assay; LIF, leukemia inhibitory factor; RSV-SEAP, Rous Sarcoma virus-secreted alkaline phosphatase; TK, thymidine kinase.
Results

The c-fms Promoter Is More Active in Macrophage Cell Lines Than in Other Cell Types. Previous studies of the human c-fms promoter present in the first intron of the gene indicated that it displayed myeloid/macrophage specificity, but the interpretation was complicated by the inclusion of the SV40 enhancer in the reporter construct (3). To examine the activity of the proximal promoter in the absence of exogenous enhancers, 5 kb of the human c-fms proximal promoter adjacent to exon 2 was tested in transient assays using luciferase reporters. The activity of the 5-kb promoter fragment was assayed and compared with the herpes simplex virus thymidine kinase (TK) promoter in the mouse macrophage cell line RAW264, the human erythroleukemia cell line K562, or in NIH 3T3 fibroblasts. Results are shown in Table 1. In either NIH 3T3 or K562 cells, the TK promoter was markedly more active (10-fold) than the c-fms proximal promoter. In contrast, the c-fms proximal promoter was 10-fold more active than TK in the macrophage cell line. These results indicate an approximate 100-fold difference in c-fms promoter activity in RAW264 cells versus the other two cell types relative to the TK promoter. The activity of the c-fms proximal promoter was also compared with the strong human β-actin promoter with the same result; it had 50-100 times the relative activity in RAW264 cells versus the other two cell types relative to the TK promoter. For these studies, progressive 5' deletion of the proximal promoter in the absence of exogenous enhancers, 5 kb of the human c-fms proximal promoter present in the first intron of the gene indicated that it displayed myeloid/macrophage specificity, but the interpretation was complicated by the inclusion of the SV40 enhancer in the reporter construct (3). To examine the activity of the proximal promoter in the absence of exogenous enhancers, 5 kb of the human c-fms proximal promoter adjacent to exon 2 was tested in transient assays using luciferase reporters. The activity of the 5-kb promoter fragment was assayed and compared with the herpes simplex virus thymidine kinase (TK) promoter in the mouse macrophage cell line RAW264, the human erythroleukemia cell line K562, or in NIH 3T3 fibroblasts. Results are shown in Table 1. In either NIH 3T3 or K562 cells, the TK promoter was markedly more active (10-fold) than the c-fms proximal promoter. In contrast, the c-fms proximal promoter was 10-fold more active than TK in the macrophage cell line. These results indicate an approximate 100-fold difference in c-fms promoter activity in RAW264 cells versus the other two cell types relative to the TK promoter. The activity of the c-fms proximal promoter was also compared with the strong human β-actin promoter with the same result; it had 50-100 times the relative activity in RAW264 cells versus the other two non-macrophage cell lines (data not shown).

A series of 5' c-fms human promoter deletions was constructed and tested in these cell lines. Results obtained with two informative 5' deletions are represented in Table 1. When tested in RAW264 cells, the c-fms proximal promoter activity slightly decreased with progressive deletion of 5' sequences. A 430-bp fragment still retained 25-50% of wild-type activity and was sixfold more active than the TK promoter. In K562 or NIH 3T3 cells, progressive 5' deletion of the proximal promoter raised the activity relative to the larger promoter constructs as well as to the TK promoter. The same results were obtained in two different luciferase reporter vectors (see Materials and Methods), indicating that the increased activity is c-fms promoter-specific, and not dependent on vector sequences. Thus, while the 430-bp proximal promoter still displayed macrophage-specificity, the effect was diminished from 100-fold (see above) to 10-20-fold relative to the TK promoter.

The apparent tissue specificity of the human promoter is not consistent with earlier work on the mouse promoter, wherein no distinction could be made between RAW264 cells and a series of murine tumor cell lines, including L929 fibrosarcoma, Lewis lung carcinoma and MOPC31C B cell lymphoma. Accordingly, a comparison of the mouse and human promoters was carried out in NIH 3T3 cells and RAW264 cells. The 3.5-kb mouse c-fms promoter was approximately five times more active than the 5-kb human promoter in both cell types. Nevertheless, there was the same apparent tissue-specificity in comparison with the TK promoter (Fig. 1) arguing that there is no fundamental difference between the mouse and human genes. The relatively high activity of the murine c-fms promoter in macrophages has been confirmed in WR19M and PU5/1.8 murine macrophage cell lines (our unpublished observations) and parallels studies of Zhang et al. (15) of the human promoter in human cell lines.

To identify critical cis-acting elements of the c-fms promoters, we concentrated on sequences located more proximal to the gene which appear critical for expression in both the mouse and human genes. An alignment of the mouse and human promoters is presented in Fig. 2 A. The number is indicated relative to the start codon, because in both species there are multiple transcription start sites. Two types of consensus sequences present in this region of the promoter were the focus for the studies presented below. In light of earlier findings showing that c-ets-2 and PU.1 can trans-activate the mouse promoter (14) one focus of interest was purine-rich ets-like binding sites. Both mouse and human promoters contain multiple repeats of sequences containing the ets core. It is difficult to choose an optimal alignment of the mouse and human proximal promoters containing these sequences, few.

Table 1. CSF-1R Promoter Activity in Macrophage vs. Non-macrophage Cell Types

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CSFR-5000</th>
<th>CSFR-900</th>
<th>CSFR-430</th>
<th>TK</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAW 264</td>
<td>36.3 (2.5)*</td>
<td>29.7 (2.5)</td>
<td>18 (2.2)</td>
<td>2.9 (0.14)</td>
</tr>
<tr>
<td>Ratio: TK</td>
<td>12.5†</td>
<td>10.2</td>
<td>6.2</td>
<td>1</td>
</tr>
<tr>
<td>K562</td>
<td>32.7 (8.7)</td>
<td>45.3 (17.6)</td>
<td>109.3 (17.9)</td>
<td>314.7 (96.3)</td>
</tr>
<tr>
<td>Ratio: TK</td>
<td>0.10</td>
<td>0.14</td>
<td>0.35</td>
<td>1</td>
</tr>
<tr>
<td>NIH 3T3</td>
<td>0.93 (0.53)</td>
<td>0.67 (0.21)</td>
<td>4.8 (1.2)</td>
<td>12.4 (1.05)</td>
</tr>
<tr>
<td>Ratio: TK</td>
<td>0.08</td>
<td>0.05</td>
<td>0.38</td>
<td>1</td>
</tr>
</tbody>
</table>

* Relative luciferase activity of each of the promoter reporters in the indicated cell lines. Each value is the average of three independent experiments performed in duplicate. The numbers in parentheses are the SD.
† Ratio of the relative luciferase activity of the CSF-1R promoter constructs to the TK promoter in the respective cell line.
since the mouse contains two apparent insertions relative to the human sequence, a GT repeat and a string of As. Attention has been focused for the purpose of this study on a conserved purine-rich region around -150 in the human and -130 in the mouse relative to the start codon (Fig. 2 A). This is downstream of the site studied by Zhang et al. (15) around -170 in the human (Fig. 2 A). The -170 site does not appear to be conserved in the mouse (Fig. 2 A).

Binding sites for the transcription factor c-myb were the second focus of interest. A perfect c-myb consensus binding site is identified in the human promoter around -375 relative to the start codon (Fig. 2 A), and we have demonstrated that this sequence is recognized by recombinant c-myb (data not shown). Based upon the detailed binding studies of Tanikawa et al. (43) the consensus core binding site for myb is AAAGCAGGG but maximal binding requires a 16-18 bp sequence. Earlier studies by Sakura et al. (44) identified low affinity myb sites (MBSII) in the SV40 early promoter. These sites had single bp mismatches within the core sequence but additional matches in sequences flanking the core. Multimers of MBSII were shown to act as myb-responsive repressor elements. Both the mouse and human c-fms promoters contain repeated weak myb binding sites concentrated in the same 60-80-bp region. These sites are annotated on Fig. 2 A and aligned with the MBSII sequence in Fig. 2 B. The sequences appear conserved between species and in one region overlap a perfectly conserved API site (TGACTCA).

ets Family Transcription Factors Can trans-Activate the c-fms Promoter. To confirm evidence of trans-activation of the mouse c-fms promoter by ets factors (14), we cotransfected the human c-fms reporters represented in Table 1 with expression vectors for ets-1, ets-2, or PEA3. The same general outcome was obtained for all promoter reporters tested, but only the results for the -430 proximal promoter construct are displayed in

Figure 1. Comparison of the activities of the mouse and human c-fms promoters in RAW264 and NIH 3T3 cells. Cells were transfected by electroporation (Materials and Methods) with 10 μg of either pGL2-3.5fms (mouse 3.5 kb flanking sequence), pCSFR-5000 (human 5.0 kb flanking sequence), or pTK-luciferase (control). After 24 h, cells were harvested and assayed for luciferase activity as described. Results are the average of three independent experiments ± SD.

Figure 2. Sequences of the human and mouse c-fms proximal promoters. (A) Mouse and human c-fms promoter sequences have been reported previously (3, 4). Potential PU-ets sites are identified based upon purine-rich sequences containing the 5'-GGAA-3' or 5'-GGAT-3' core on either strand. Potential weak c-myb sites (43) are underlined in bold. Arrows indicate identified transcription start sites (3, 4). Dotted underline indicates the -170 functional PU-box in the human promoter (15). These sequence data are available from EMBL/GenBank/DDBJ under accession number X14720 (human). (B) Potential weak binding sites for c-myb in the human and mouse promoters are aligned with the consensus, strong (MBSI) and weak (MBSII) myb sites (43). Mismatches with the myb consensus are in bold. These sequence data are available from EMBL/GenBank/DDBJ under accession number S62219 (mouse).
Fig. 3 A. The data demonstrated that ets-1 or ets-2 were equally effective at trans-activating the c-fms proximal promoter. PEA3 did not activate these reporters, indicating a selectivity in factor-promoter interaction. Transactivation occurred in NIH 3T3 fibroblasts, as well as in RAW264 cells. The average activation was eightfold in the former cell type and about three- to fourfold in the latter. The TK or actin promoters were not transactivated by the ets expression vectors (data not shown). Hence, the human promoter, like the mouse promoter, is responsive to ets transcription factors. The responsiveness of the human (15) and mouse (14) promoters to the macrophage-specific ets-related factor PU.1 has been described previously. Fig. 3 B demonstrates that the human c-fms proximal promoter (−430) is transactivated by coexpression of PU.1 in NIH 3T3 cells, as it is in HeLa cells (15). PU.1 increased proximal promoter activity by six- to sevenfold.

The alignment of sequences in Fig. 2 A suggests that the ets/PU.1-like sequences around 150 in the human and −130 in the mouse are conserved and might play a role in the tissue specificity and ets/PU.1 responsiveness exhibited by this promoter in the transient assays. To test this possibility, G-residues in the consensus sequence necessary for ets factor recognition (5, 6) were mutated in the context of the −430 luciferase reporter construct and the activity of the mutated proximal promoter was assayed (Fig. 3C, 430-WT vs. 430-M1). The experiments revealed that in RAW264 cells the 430-M1 promoter had 20% of the activity of the 430-wt promoter. The point mutations rendered the promoter only twofold more active than the TK promoter in RAW264 cells. The absolute activation of the proximal 430-M1 promoter by c-ets-1 was largely abrogated, with 10% of the wild-type activity remaining. In terms of fold stimulation over basal activity by c-ets-1 the 430-M1 mutant was stimulated twofold, vs. fivefold stimulation of 430-WT. Therefore, the ets-related sequences were critical for the basal activity of this promoter in RAW264 cells, and were also necessary for efficient transactivation by c-ets-1.

Because PU.1 is already expressed in RAW264 cells, and overexpression causes repression (14) the effect of the mutation of the −150 element was tested in NIH 3T3 cells (Fig. 3 B). The mutation caused a lesser reduction in basal activity in NIH 3T3 cells than in RAW264 cells (40% decrease in basal activity in NIH 3T3 cells vs. 70% in RAW264 cells).

Figure 3. Activation of the c-fms proximal promoter by c-ets-1, c-ets-2, and PU.1 and the role of the putative −15- response element. (A) CSFR-430 reporter was cotransfected with expression vectors for ets-factors into either NIH 3T3 cells or RAW 264 cells, as indicated, by calcium phosphate-mediated transfection (NIH 3T3 cells) or electroporation (RAW 264 cells). Rous sarcoma virus-secreted alkaline phosphatase (RSV-SEAP) was included as internal control. For the latter, 20 μg of DNA was added per experiment, consisting of 15 μg of reporter, 1 μg of transactivator expression vector, 1 μg of RSV-SEAP, and 3 μg of carrier plasmid vector DNA. The luciferase activity was assayed 48 h posttransfection. (B) 10 μg of CSFR-430 (WT) or a plasmid mutated in the −150 ets site (5) was cotransfected into NIH 3T3 cells with 1 μg of either ets-1 expression plasmid PU-pECE or the host plasmid (pECE) by electroporation (Materials and Methods). Luciferase activity was assayed after 24 h. (C) 20 μg of reporter plasmids containing either wild-type CSFR-430 or the mutated CSFR-430 (M1) promoter was transfected into RAW264 cells with 1 μg of either parent plasmid (basal) or the ets-1 expression vector (open bars) 1 μg of RSV-SEAP, and 3 μg of carrier plasmid. Luciferase activity was assayed after 48 h. The above experiments are the average of three separate experiments, each performed in duplicate and the error bars indicate the SD.
activity vs. 80%). Additionally, the 430-M1 reporter displayed a 60% reduction in PU.1 responsiveness, to 2.5-fold stimulation over the basal activity.

**EMSA of Nuclear Proteins That Interact with the −150 Element of the Human c-fms Promoter.** The expression vector used for c-ets-2 placed an influenza virus–derived epitope tag at the NH₂ terminus of the transcription factor (39), thus allowing exogenous c-ets-2 to be distinguished from the endogenous protein. The epitope-tagged c-ets-2 was employed to detect interactions of this protein with sequences in the CSF-1R proximal promoter. In EMSA using the wild-type ets-oligonucleotide, an increase in a specific DNA binding complex was observed in extracts that contained overexpressed c-ets-2 (Fig. 4 A, lane 3 vs. lane 1, open arrow). Competition with excess homologous oligonucleotide abolished the formation of this complex (Fig. 4 A, lane 4), while competition with the oligonucleotide containing point mutations in the ets-related sites (see Materials and Methods) did not affect complex formation (Fig. 4 A, lanes 5 and 6). Inclusion of monoclonal antibody 12CA5-F, specific to the epitope-tag (39) that marked the exogenous c-ets-2, in the EMSA resulted in a supershift of this complex (Fig. 4 B, lanes 8–10, closed arrow). Increasing the amount of specific antibody in the EMSA caused an increase in the supershifted complex, while having no effect on the endogenous c-ets-2 complex formed when mock-transfected extracts were used (Fig. 4 A, lanes 2–4). Monoclonal antibodies directed against the yeast transactivator gal4 or c-myb had no effect on complex formation (Fig. 4 A, lanes 11 and 12, respectively). A complex with identical mobility to the c-ets-2 complex shown in Fig. 4 was also found in nuclear extracts from RAW 264 cells, which express c-ets-2 mRNA (data not shown).

Zhang et al. (15) suggested that PU.1 could not bind to the −150 c-fms ets-like sequence we have studied. Fig. 5 compares the mobility of RAW264 nuclear proteins that bind this sequence to recombinant PU.1 expressed in E. coli. One RAW264 nuclear protein bound to the wild-type, but not mutant M1 sequence. This protein conformed with recombinant PU.1, which also bound specifically to the c-fms sequence, and with a protein that binds the SV40 PU-box recognition site for PU.1 (Fig. 5, lanes 2 and 3, compared with lanes 6 and 7). The identity of PU.1 as the only nuclear protein in RAW264 cells that binds the SV40 PU box has previously been confirmed by antibody supershift and the pattern of partial protease cleavage (14). Hence, both native and recombinant PU.1 are able to bind specifically the −150 c-fms purine-rich sequence and the mutations that reduce activity of the promoter (Fig. 3 B) abolish PU.1 binding (Fig. 5, lanes 4 and 5).

c-fms mRNA Expression and Promoter Activity Increase during M1 Cell Differentiation. In view of the apparent presence of candidate c-myb repressor sites in the mouse and human c-fms promoters (Fig. 2 A and B), we investigated the activity of the promoters in the M1 murine myeloid leukemia line. This line undergoes macrophage differentiation in response to LIF in parallel with a decline in c-myb mRNA expression (45). Fig. 6 shows the time course of induction of c-fms mRNA in M1 cells undergoing differentiation in response to LIF. c-fms mRNA first became detectable after 2 d of LIF treatment, and maximal expression occurred by day 3 after LIF treatment. A similar time course of induction was reported previously for other macrophage-specific genes such as lysozyme (45). The level of c-myb mRNA actually increased in control cells during the time course of the experiment, probably reflecting an increase in growth rate as the cells were diluted. Within 24 h of addition of LIF, c-myb mRNA levels were decreased and became undetectable after 48 h of LIF treatment (Fig. 6). Thus, the disappearance of c-myb mRNA correlated with the expression of c-fms mRNA.

When the 3.5-kb murine c-fms promoter was assayed by transient transfection in M1 cells, the activity was not significantly greater than that of the promoterless pGL2-basic

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**Figure 4.** Interaction of c-ets-2 with the purine-rich sites in the CSF-1R proximal promoter. (A) Whole cell extracts of the COS-7 cells transfected with pCGN (empty expression vector, lanes 1 and 2) or pCGN-c-ets-2 (epitope-tag vector, lanes 3–6) were incubated with a double-stranded ⁷P-labeled probe containing the ets-related sequences highlighted in Fig. 2 A. In the reactions loaded into lanes 2 and 4, a 20-fold molar excess of homologous oligonucleotide was included, whereas for those in lanes 3 and 5, a 20- or 40-fold molar excess, respectively, of the corresponding mutated oligonucleotide (see Materials and Methods) were included. The open arrow indicates the position of the specific c-ets-2 complex. (B) Whole cell extracts of the COS-7 cells transfected with pCGN (empty expression vector, lanes 1–6) or pCGN-c-ets-2 (epitope-tag vector, lanes 7–12) were incubated with a double-stranded ⁷P-labeled probe containing the ets-related sequences highlighted in Fig. 1 B (see Materials and Methods). Lanes 1 and 7 had no addition. Lanes 3–5 and 8–10 had increasing amounts of the affinity-purified monoclonal antibody specific to the epitope tag, 12CA5-F, added (0.25 μg, 0.5 μg, 1 μg, respectively). Lanes 5 and 11 had 2 μg of a monoclonal antibody specific for the yeast transactivator gal4 added (a gift from Ivan Sadowski, University of British Columbia, Vancouver, Canada) whereas lanes 6 and 12 had 2 μg of monoclonal antibody directed against mouse c-myb (type I, UBI) added. The extracts were preincubated for 10 min with monoclonal antibodies before addition of the probe. The open arrow indicates the position of the specific c-ets-2/DNA complex and the closed arrow represents the super-shifted c-ets-2/DNA complex.

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negative control plasmid, a relative activity even lower than in NIH 3T3 cells. Differentiation of the cells in LIF led to a 10–20-fold induction of c-fms promoter activity (Fig. 7).

The activity of other control promoters tested also increased, though only by two- to threefold, perhaps reflecting alterations in transfection efficiency. The results are consistent with the data indicating that the c-fms promoter contains elements that can direct monocyte/macrophage-specific expression of reporter genes, and that c-myb may play some role in this regulation.

The latter possibility was tested directly in RAW264 cells (Fig. 8 A). Nuclear extracts prepared from RAW264 cells had no c-myb DNA binding activity as determined by EMSA, and c-myb could not be detected by Western blotting (data not shown). Transient expression of exogenous c-myb led to a 70–80% reduction of either the −5,000 or −430 human c-fms promoter activity (Fig. 8 A). Further, neither v-myb or a COOH-terminal truncated version of c-myb that lacks the last 199 amino acid residues were able to mimic this repression. A c-fms promoter reporter truncated at position −367, just downstream of the candidate c-myb binding sites (Fig. 2 A), was not affected by expression of c-myb proteins. EMSA and supershift assays demonstrated that the perfect c-myb consensus sequence located at position −383 in the human promoter (see Fig. 2 B) was recognized by recombinant c-myb protein (data not shown). c-myb was equally effective as a repressor of the full-length 3.5-kb murine promoter, causing a 70% reduction in promoter activity (Fig. 8 B). Deletion of the proximal murine promoter to 0.3 kb, which again removes the putative c-myb sites, abolished the repression (Fig. 8 B). The repressive action of c-myb was specific to the fms promoter. When a reporter containing 5 myb-consensus sites upstream of a TK promoter (20) was tested, v-myb and both c-myb constructs trans-activated expression, and, as expected (37, 38), v-myb or truncated c-myb were stronger trans-activators than full-length c-myb (Fig. 8 A).

The relationship between c-ets-1 or c-ets-2 activation and c-myb repression was tested using the 430-WT human fms promoter (Fig. 8 C). c-myb expression was able to repress ets-factor stimulation of c-fms proximal promoter activity in RAW264 cells. As above, COOH-terminal truncated c-myb (Fig. 8 C) or v-myb (data not shown) were ineffective repressors. When the effect of c-myb on the c-fms proximal promoter was tested in NIH 3T3 cells (Fig. 8 D), there was no repression of the basal promoter activity of the CSFR-5000 reporter, but instead slightly (2.5-fold) stimulated promoter activity. As demonstrated above, c-ets-2 transactivated the proximal promoter in NIH 3T3 cells. In contrast to the
results in RAW264 cells, a combination of c-ets-2 and c-myb was not significantly different than c-ets-2 alone (Fig. 8 D). The same results were obtained in the K562 cell line (data not shown). These results demonstrate a cell-type specificity for c-myb action.

Discussion

The data presented here indicate that sequences in the 450-bp flanking the c-fms start codon of both the mouse and human c-fms genes can direct macrophage-specific expression of reporter genes. This conclusion is not incompatible with a previous study on the mouse promoter that demonstrated that attenuation of transcription was a crucial point of regulation (4). The nonmacrophage cell lines used in that study were all tumorigenic. We have shown subsequently that the murine c-fms promoter is activated by serum and by malignant transformation (Hume, D. A., unpublished observations). Hence, the choice of controls is important to identification of tissue-specific cis-acting elements by transient transfection. Ultimately, the elements required for specific expression of c-fms will need to be delineated in transgenic mice.

In studying the role of purine-rich ets-like sequences as cis-acting elements in the human c-fms promoter, we have con-
mented upon an element around position −150, downstream of that previously studied by Zhang et al. (15) around −170. This element is apparently conserved in the mouse promoter (Fig. 2, A). Mutation of the ets-related sites at around position −150 led to a substantial reduction of the c-fms proximal promoter activity, demonstrating that these sequences are necessary for maximal transcription in RAW264 macrophages. Either c-ets-1 or c-ets-2 directly activated the promoter via these sequences in a cell-type independent manner and an epitope-tagged form of c-ets-2 recognized the critical sequences. Whereas the expression of c-ets-1 is thought to be lymphocyte-specific (6), c-ets-2 is expressed in macrophages (14) and is a candidate as one factor necessary for c-fms proximal promoter activity. PU.1 also binds the −150 element, in contrast to the findings of Zhang et al. (15) who reported that this site was not recognized by PU.1. Further, mutation of the −150 downstream element reduced transactivation by PU.1 in NIH 3T3 cells. We conclude that multiple members of the ets family can interact with the −150 element, and that the −170 PU/ets site (15) is not the only such functional element in the human c-fms promoter. Both the −150 element described here and the −170 element appear to be necessary, but not sufficient, for human c-fms promoter specificity.

PU.1 and c-ets-2 probably mediate their effects on c-fms gene expression by interacting with other proteins. In our hands, a PU-box multimer has no effect on the activity of a basal promoter when tested in RAW264 cells (Ross, I. L., C. J. K. Barnett, and D. A. Hume, unpublished observations). Similarly PU.1 expression in B cells did not transactivate a PU-box multimer from the IκB enhancer unless the adjacent binding site for another factor, NF-EM5 was included (9). PU.1 binds specifically to the TATA-binding protein in vitro (46). Zhang et al. have previously suggested that PU.1 may recruit TATA binding protein to TATA-less promoters such as that of c-fms (15). The −150(h)/−130(m) PU.1 binding site we have studied is placed more appropriately than the more distal −170 for such a function. Like a typical TATA box, it is around 30–40 bp upstream relative to the major transcription start sites of the mouse and human, and unlike the −170 site appears to be conserved between the two species (Fig. 2, A).

Our studies suggest that c-fms is subject to negative as well as positive regulation during myeloid differentiation. The repression of the human c-fms promoter by c-myb was mediated by sequences between −430 and −367, which contains multiple c-myb sites conserved between mouse and human promoters. The less refined deletion of the mouse promoter supports the idea that the elements are conserved functionally, in that the 0.3-kb mouse promoter was c-myb unresponsive. Full-length c-myb was a repressor of the c-fms proximal promoter, but neither a COOH-terminal truncated version of c-myb nor v-myb, which also lacked the COOH-terminal domain, functioned as an efficient repressor. The COOH-terminal domain has been shown to be a cis-acting negative element that blocks c-myb trans-activation and transforming activity (36, 37). Our results suggest an additional function for this domain as a site involved in the trans-repressive activity seen in RAW264 cells. This conclusion is quite different from the results of Sakura et al. (44). In their studies of the myb responsive elements of SV40, the weaker MBSII sites functioned only as cis-acting repressor elements in a cell-type independent manner and the COOH terminus of c-myb was not required for repression.

When activating transcription, c-myb action is context dependent hinging on interactions with other transactivators (31–34). The repression of the c-fms proximal promoter by c-myb is also context dependent, since when assayed with a heterologous promoter the human c-myb–responsive element can function as an enhancer (data not shown). Additionally, c-myb repression of the human c-fms proximal promoter did not occur in NIH 3T3 cells. Thus, interaction(s) with distinct transactivators are probably crucial for repression of the c-fms promoter by c-myb as well. Since c-myb is already known to interact with ets factors in transactivation (32) and c-myb blocked activation of the c-fms proximal promoter by c-ets-1 or c-ets-2 in RAW264 cells, ets factors present attractive targets for the negative action of c-myb. In this context, the parallel between myb and PU.1 is of some interest. PU.1 is the product of the Spi-1 protooncogene, which was identified as a major site of integration of spleen focus forming virus (47). Like c-myb (48), PU.1 can prevent erythropoietic differentiation (47). The possibility that myb can interact with PU.1 is worthy of further consideration.

Knockout experiments in transgenic mice demonstrate the critical role of the c-myb protooncogene product in the expansion and maintenance of hematopoietic stem cells (22). Our results suggest that, in addition to activating genes required for stem-cell growth and survival, a significant effect of c-myb during the early stages of hematopoiesis may be to repress the expression of key regulatory genes necessary for the terminal stages of differentiation, such as c-fms. The transcriptional repressor activity of c-myb may provide a checkpoint ensuring that precursor cells cannot prematurely enter terminal differentiation pathways. The loss of this capacity from oncogenic forms of myb may contribute to their transforming potential.

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