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Expression of mRNA Encoding the Macrophage Colony-Stimulating Factor Receptor (c-fms) Is Controlled by a Constitutive Promoter and Tissue-Specific Transcription Elongation

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The gene encoding the receptor for macrophage colony-stimulating factor 1 (CSF-1), the c-fms proto-oncogene, is selectively expressed in immature and mature mononuclear phagocytes and trophoblasts. Exon 1 is expressed only in trophoblasts. Isolation and sequencing of genomic DNA flanking exon 2 of the murine c-fms gene revealed a TATA-less promoter with significant homology to human c-fms. Reverse transcriptase primer extension analysis using exon 2 primers identified multiple clustered transcription initiation sites. Their position was confirmed by RNase protection. The same primer extension products were detected in equal abundance from macrophage or nonmacrophage sources of RNA. c-fms mRNA is acutely down-regulated in primary macrophages by CSF-1, bacterial lipopolysaccharide (LPS), and phorbol myristate acetate (PMA). Each of these agents reduced the abundance of c-fms RNA detectable by primer extension using an exon 3 primer without altering the abundance of presumptive short c-fms transcripts detected with exon 2 primers. Primer extension analysis with an intron 2 primer detected products at greater abundance in nonmacrophages. Templates detected with the intronic primer were induced in macrophages by LPS, PMA, and CSF-1, suggesting that each of the agents caused a shift from full-length c-fms mRNA production to production of unspliced, truncated transcripts. The c-fms promoter functioned constitutively in the RAW264 macrophage cell line, the B-cell line MOPC.31C, and several nonhematopoietic cell lines. Macrophage-specific expression and responsiveness to selective repression by LPS and PMA was achieved by the incorporation of intron 2 into the c-fms promoter-reporter construct. The results suggest that expression of the c-fms gene in macrophages is controlled by sequences in intron 2 that act by regulating transcription elongation.

Macrophage colony-stimulating factor 1 (CSF-1) promotes the survival, proliferation, and differentiation of cells of the mononuclear phagocyte series (29). Its biological activities are mediated by binding to a plasma membrane receptor, the product of the c-fms proto-oncogene, which is a ligand-dependent protein tyrosine kinase (24). Expression of c-fms mRNA and CSF-1 binding activity is largely restricted to mononuclear phagocytes and placental trophoblasts (24). Although there has been considerable progress in understanding tissue-specific gene expression in other hematopoietic cell lineages, macrophage differentiation remains poorly understood at the level of gene transcription. The most extensive studies have been of the lysozyme gene in chickens, which is controlled by a complex interaction between macrophage-specific enhancer elements and silencers that restrict gene expression in nonmacrophages (10). The paucity of information about macrophage-specific gene expression has been attributed to the difficulty of obtaining high transfection efficiencies with primary mammalian macrophages or myelomonocytic cell lines (25), a technical obstacle that we have recently overcome in a study of the constitutive elements controlling the expression of the urokinase plasminogen activator gene in macrophages (3). The limited literature on macrophage-specific gene expression has been largely dependent upon the use of transgenic animals (2, 25), which places limitations on detailed analysis of cis-acting sequences. Preliminary characterization of the human gene encoding c-fms revealed that in the two cell types that express the gene, trophoblasts and macrophages, transcription is initiated from two separate promoters, separated by a 25-kb intron (30). A limited analysis of transcriptional regulation of the two promoters in human cell lines (20) suggested that a 550-bp segment flanking exon 2 can direct macrophage-specific expression of a reporter gene. In this report, we show that the murine c-fms gene contains a constitutive promoter and that production of full-length mRNA is controlled by sequences in intron 2 that regulate transcript elongation.

MATERIALS AND METHODS

Cell lines and cell culture. The cell lines RAW264, Lewis lung carcinoma (LLC1 or LL/2), L929 (fibrosarcoma), BALB/3T12-3 (embryonic fibroblasts), BALB/c 10ME HD.A 5R.1 (methylcholanthrene-transformed fibroblasts), BALB/c CI.7 (embryonic fibroblasts), and MOPC.31C were obtained from the American Type Culture Collection. They were maintained in RPMI 1640 plus 10% fetal bovine serum. Primary bone marrow-derived macrophages (BMDM) were produced by cultivation of murine femoral bone marrow cells in recombinant human CSF-1 (a gift from Chiron Corp.) as described previously (14, 15). Lipopolysaccharide (LPS; ReS95 from Salmonella minnesota) and phorbol myristate acetate (PMA) were obtained from Sigma.

Mouse c-fms genomic clone isolation and plasmid construction. A mouse genomic DNA library from the A17 murine T-cell lymphoma cell line in λEMBL3A (a gift from Mark

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Hogarth) was screened with a 5' restriction fragment of the murine c-fms cDNA (21) kindly provided by Larry Rorschede. A clone containing a 14-kb SalI insert was isolated, the restriction map of which was consistent with the identity of this clone as the SalI genomic DNA fragment which encompasses the 5' end of the gene (9). A 7-kb ApaI fragment bracketing the 5' end of the gene was subcloned into pBluescript (Stratagene) to yield pXYfms1, and subfragments were sequenced by the dyeoxy-chain termination method, using a Pharmacia T7 polymerase kit and double-stranded plasmid templates. The 3' ApaI site in this clone is at position 134 in the murine cDNA sequence (21). The restriction map of this fragment is presented in Fig. 7, in the context of the analysis of reporter constructs. Chloramphenicol acetyltransferase (CAT) reporter constructs were generated by subcloning the desired restriction fragments from the ApaI genomic DNA fragment in pBluescript into the multiple cloning site of the vector pCAT-Basic (Promega). The positive control vector pHβAPr-CAT, a gift from Peter Gunning, contains a 4.3-kb EcoRI-Abl1 fragment of the human β-actin promoter linked to the CAT gene. An additional set of reporter constructs was constructed in the luciferase reporter gene plasmid pGL2-Basic (Promega). This vector has the advantage of both increased sensitivity and a low nonspecific background due to the presence of a polyadenylation signal, to prevent read-through transcripts from the vector, placed upstream of the multiple cloning site. The desired restriction fragments from the ApaI genomic DNA clone were also cloned into the multiple cloning site of this vector. In the case of the intron-containing c-fms plasmid, pGL6.7fms, the c-fms genomic DNA was removed from pXYfms1 as an SpeI fragment and inserted into the same site in pGL2-Basic. The 5' SpeI site in this case is approximately 50 bp into the c-fms promoter sequence, while the 3' SpeI site is in the pBluescript multiple cloning site. Plasmid pGL2-Control, containing the simian virus 40 (SV40) early promoter and enhancer, was used in some experiments. Because this vector displayed some apparent tissue specificity, another control vector was produced by inserting the EcoRI-Abl1 fragment of the human β-actin promoter (see above) into the multiple cloning site of pGL2-Basic. This plasmid is referred to as pGL-HβAPr.

RNA extraction, RNA protection, and primer extension analysis. RNA was isolated by the method of Evans et al. (8). Briefly, cells were lysed by shearing in 8 M guanidine-HCl–0.3 M sodium acetate–1% sarcosyl. After centrifugation, the supernatant was ethanol precipitated, and the pellet was suspended in 8 M guanidine–0.3 M sodium acetate, reprecipitated, washed twice with 70% ethanol, and dissolved in Tris-EDTA buffer. Primer extension was performed exactly as described by Sambrook et al. (22) except that N-2-hydroxyethylpiperezine-N'-2-ethanesulfonic acid (HEPES) buffer was substituted for piperezine-N,N'-bis(2-ethanesulfonic acid) (PIPES). Superscript RNase H- Moloney murine leukemia virus reverse transcriptase was obtained from Bethesda Research Laboratories. The following primers were used (their positions in the c-fms gene are described in Results): primer A, 5'-TTGGAGAATCGTGCTGGCGAAC GGGCTCCACGCTGCTATT; primer B, 5'-ATGCAA AACGTGGCAGGACAGGAGGAGAGGGCC; primer E, 5'-CCAGTTCGTCGACTGA CAGGGGCCCCTGACCATG; and primer F, 5'-AGGGCA TCACATGCGAGAGGAGCTGTGTGACTAGTTGCAACACT CCCC.

At the end of the reaction, 20 μl of 1 M NaOH was added to the 30-μl incubation, and the RNA template was hydrolyzed for 15 min at 65°C before neutralization with 20 μl of 1 M HCl plus 20 μl of 1 M Tris-HCl (pH 7.6). The transcribed DNA was recovered by ethanol precipitation and separated by electrophoresis on 6% polyacrylamide–8 M urea sequencing gels in parallel with a sequencing ladder generated by using the same primer and pxyfms1 as a template.

For RNase protection assays, a 410-bp c-fms genomic DNA fragment (XhoI [−203] to SmalI [+207]) or a 200-bp 5' cDNA fragment (EcoRI in the host vector to BsrEII at bp 186 [21]) was cloned into pBluescript. A 32P-labeled antisense RNA probe was synthesized by using T3 RNA polymerase. For dot blot hybridization, a sense probe was also synthesized by using T7 RNA polymerase and treated identically. The reaction mixture contained 2 μg of template DNA in 100 μl of reaction buffer (40 mM Tris-HCl [pH 7.6], 6 mM MgCl2, 2 mM spermidine, 5 mM NaCl, 10 mM dithiothreitol, 200 μg of bovine serum albumin per ml, 500 μM ATP, CTP, and GTP, 25 μM UTP, 10 μl of [α-32P]UTP [10 μCi; 800 Ci/mmol; New England Nuclear]). The reaction was terminated by addition of DNase I, the products were separated on 5% polyacrylamide gels, and the labeled probe band was excised and eluted overnight in 50% deionized formamide. The probe (50,000 cpm) was added to 10 μg of DNase I-treated RNA, precipitated in 70% ethanol, and redissolved in 20 μl of 4 mM HEPES (pH 6.4)–0.1 mM EDTA–40 mM HCl–80% deionized formamide. After heating to 85°C for 15 min, the mixture was cooled to 45°C and allowed to hybridize overnight. Then 350 μl of RNase buffer (10 mM Tris-HCl [pH 7.5], 5 mM EDTA, 300 mM NaCl, 0.4 μg of RNase T1, 8 per ml, μg of RNase A per ml) was added, and the mixture was incubated for 30 min at 37°C. The reaction was stopped by addition of 20 μl of 10% sodium dodecyl sulfate (SDS) plus proteinase K (final concentration, 12.5 μg/ml) and incubation for 30 min. Following phenol-chloroform extraction and ethanol precipitation in the presence of 20 μg of tRNA, the protected bands were separated on 8% polyacrylamide sequencing gels.

Transient transfection. Transient transfections and CAT assays were carried out as previously described (3). Briefly, 5 × 105 cells in 250 μl were transfected by electroporation at 750 V/cm (0.4-cm cuvettes) and 960-μF capacitance in RPMI 1640–10% fetal calf serum at room temperature and returned immediately to culture at 37°C. After 24 h for luciferase or 48 h for CAT assays, cells were harvested and assayed for reporter gene activity. The luciferase activity was assayed by using reagents supplied by Promega and measured with a Berthold luminometer. Activities were normalized to cell protein assayed by using a Bradford microprotein assay with reagents supplied by Bio-Rad.

RESULTS

c-fms promoter sequence. Figure 1 shows the DNA sequence of the mouse and human c-fms genes flanking exon 2. Because of the heterogeneous transcription initiation sites identified below, the two sequences are numbered relative to the initiation codon. Between −160 and −110, the alignment is tenuous, as both species have an extended polypurine tract which in the mouse includes 24 consecutive A residues. The alignment between the two species is also interrupted around −250 by the insertion in the mouse of a short GT repeat that is absent from the human gene. Beyond the conserved region shown, there is no obvious homology between the next 250 bp of mouse sequence and 600 bp of human sequence (not shown). In this region, the human gene contains an Alu repeat (20). The most conserved parts of the
c-fms promoters of the mouse and human genes contain several sites similar to the binding sites of the ubiquitous CCAAT box-binding proteins (Fig. 1), but none fits perfectly the consensus sequence for CBP-1, CBP-2, or NF-1 (6). The c-fms promoters in both species contain multiple polypurine and polypyrimidine strings containing the 5'-GAGA-3' recognition site of the ets family of transcription factors (31), including two copies in each species of the PU box (5'-GAGGAA-3'), the binding site for the transcription factor PU.1, which is restricted to B cells and macrophages (17).

One other feature of interest is the multiple AP1-like (5'-TGACTCA-3') and AP2-like (5'-CCAGGC-3') sites in both species, both of which might confer phorbol ester respon- siveness (5). Finally, the murine c-fms promoter contains the TATA-like sequence 5'-TTATTTATGC-3' at −252 (Fig. 1), but this sequence does not appear to be conserved in the human gene.

Reverse transcriptase primer extension and RNase protection analysis of c-fms mRNA in macrophages and nonmac- rophages. To determine the transcription start site of the mouse c-fms promoter flanking exon 2, we performed reverse transcriptase primer extension analysis using DNase I-treated RNA from murine BMDM (Fig. 2). Primer extension using a 40-bp oligonucleotide (primer A) complementary to the 5' end of the reported murine cDNA sequence (~75 in Fig. 1) (21) revealed numerous candidate transcription start sites (Fig. 2A). No bands were observed when nucleotides were omitted from the reverse transcriptase reaction (Fig. 2A) or when tRNA was used as a template (not shown). The major candidate transcription start sites are indicated in Fig. 1. While some of them correlate exactly with those identified in human monocyte-like cells (20), others extend further upstream. The same pattern of transcription start sites was observed when RNA from the macrophage cell line RAW264 was used (Fig. 2A). Surpris- ingly, identical primer extension products at comparable abundance were also produced with use of RNA from nonmacrophage cell lines such as the B-lymphocyte cell line MOPC.31C (Fig. 2A). To eliminate the possibility of artifact, the primer extension was repeated with use of a second 40-bp oligonucleotide (primer B) which hybridized to the end of the second exon, approximately 80 bp 3' of primer A. Again, the same products at comparable abundance were obtained with use of RNA from primary macrophages, RAW264 cells, and MOPC.31C B cells (Fig. 2B).

Evidence for this lack of specificity was extended to nonhematopoietic cells, L929 fibrosarcoma (Fig. 2B) and Lewis lung carcinoma (not shown). Finally, when primer E, complementary to the 5' end of exon 3, was used, extension products were generated from primary macrophage or RAW264 RNA (Fig. 2C) but not from MOPC.31C, Lewis lung carcinoma (Fig. 2C), or L929 (not shown) RNA. This finding suggested that c-fms transcripts are initiated in nonmacrophages but terminate before the beginning of exon 3.

c-fms mRNA is down-regulated in macrophages by the ligand, CSF-1, and by bacterial LPS and phorbol esters (PMA) (11, 23). The actions of LPS are associated with cessation of c-fms-specific transcription in nuclear run-on transcription assays (11). We confirmed that each of these agonists down-regulated c-fms mRNA in BMDM and RAW264 cells by Northern (RNA) analysis (data not shown, but see also Fig. 6). Figure 3 shows the result of primer extension analysis with RNA from BMDM starved of CSF-1 (Fig. 2B) and by bacterial LPS and phorbol esters (Fig. 2C) but not from MOPC.31C, Lewis lung carcinoma (Fig. 2C), or L929 (not shown) RNA. This finding suggested that c-fms transcripts are initiated in nonmacrophages but terminate before the beginning of exon 3.

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rich elements containing dyad symmetry which might potentially form stable stem-loops in transcribed RNA or single-stranded DNA (27). The intronic sequences of both the mouse and human c-fms genes contain extended GC-rich repeats. Potential stem-loops formed by these repeats are shown in Fig. 4 and compared with the TAR region of the human immunodeficiency virus, a known stable secondary structure (27).

Primer extension was repeated by using a primer (primer F) based upon the intron 2 sequence around the predicted stem loop in Fig. 4. Identical primer extension products were detected in all cell types examined, except BMDM starved of CSF-1 to maximally up-regulate c-fms mRNA (Fig. 5). In these cells, the level of intron-containing RNA was much lower but was increased by each of the agonists, LPS, PMA, and CSF-1, shown to down-regulate full length c-fms mRNA (Fig. 3). The data suggest that the level of intron-containing transcripts varies inversely with the level of full-length c-fms transcripts and are consistent with the existence of a site of selective transcription elongation within intron 2.

In an attempt to identify the sites of transcription attenuation in intron 2 and to independently validate the primer extension results, we performed RNase protection studies. c-fms genomic DNA extending from –203 to +210 was cloned into pBluescript, and a radiolabeled RNA probe was generated by using T3 RNA polymerase. Both BMDM and RAW264 RNA protected the labeled probe against RNase digestion, producing multiple protected bands (Fig. 6A). The position of these bands relative to the splice donor site at the end of exon 2 correlated precisely with the clustered transcription start sites identified by primer extension in Fig. 2. RNA obtained from BMDM treated with CSF-1, LPS, or PMA was less effective in producing all of the protected bands, indicating that the abundance of each of the bands correlates with the abundance of full-length c-fms mRNA.

Because of the extensive GC-rich inverted repeats in exon 2 and the beginning of intron 2, we reasoned that internal stem loops in the intron-containing truncated transcripts could be interfering with efficient hybridization. To eliminate these potential structures from the probe, we prepared a new probe template by subcloning the 5' end of murine c-fms cDNA (21) to the BstEII site at bp 186 into pBluescript. With labeled antisense RNA transcribed from this segment used.
as a probe, RNA from BMDM or RAW264 cells generated the expected 186-base protected band, but it was still impossible to detect any protected bands when nonmacrophage RNA was used (data not shown).

The only way to eliminating secondary structures from the transcripts is to denature and immobilize them. Accordingly, the 5' c-fms antisense RNA probe was used in a dot blot hybridization on macrophage and nonmacrophage RNA bound to nitrocellulose membranes. The result (Fig. 6B) indicates that macrophages and nonmacrophages do indeed contain approximately equal levels of transcripts that hybridize to the 5' end of c-fms. Furthermore, as suggested by the primer extension data in Fig. 3, CSF-1, LPS, and PMA had no significant effect on the abundance of transcripts detected with the 5' c-fms probe. The reasons why short transcripts cannot be detected in nonmacrophages by RNase protection are considered in Discussion.

FIG. 3. Effects of CSF-1, LPS, and PMA on c-fms transcription attenuation in BMDM. BMDM were washed and incubated overnight in the absence of CSF-1 to maximally induced c-fms mRNA. They were then incubated for 4 h with CSF-1 (1,000 U/ml), LPS (100 ng/ml), or PMA (10^{-7} M) prior to RNA isolation. RNA (50 μg) from each treatment was incubated with radiolabeled primer B (end of exon 2) or primer E (beginning of exon 3) and extended with reverse transcriptase. The extension products were separated on a sequencing gel in parallel with a sequencing reaction. Quantitation was achieved by scanning the entire gel with an AMBISS Radioanalyser and counting the total radioactivity in the extension products in each lane. The results expressed as total counts per minute are displayed below each lane.
CAT gene was fused at -57. Treatment of the RAW264 cells transfected with the reporter constructs with PMA had no effect on CAT activity (Fig. 7a). The activities of long (pFMS3.5CAT) and short (pFMS0.3CAT) c-fms reporter constructs were also tested in nonmacrophages, using a β-actin promoter-CAT construct as a transfection control. The relative activities of the two c-fms constructs did not distinguish RAW264 cells from the B-cell line MOPC.31C or any of the nonhematopoietic murine tumor cell lines tested (Fig. 8).

To address the role of intron 2 in generating tissue specificity, the c-fms genomic DNA fragments were transferred to the luciferase reporter construct, pGL2-Basic. Apart from the availability of a more convenient set of

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**FIG. 5.** Detection of RNA containing sequences homologous to c-fms intron 2 by reverse transcriptase primer extension. DNase-treated RNA was prepared from each of the cell lines indicated and from BMDM treated with CSF-1, LPS, or PMA as indicated in the legend to Fig. 3. RNA (50 μg) was hybridized to radiolabeled primer F, which is based upon the sequence in the vicinity of the potential stem-loop early in murine c-fms intron 2 (Fig. 4), and extended by using reverse transcriptase. The extension products were separated on a sequencing gel in parallel with a sequencing ladder generated by using the same primer. Quantitation was achieved by scanning the entire gel with an AMBIS Radioanalyser and counting the total radioactivity in the extension products in each lane. The results (expressed as total counts per minute) are displayed below each lane.

**FIG. 6.** (A) RNase protection analysis of the 5' ends of c-fms transcripts expressed in macrophages. RNase protection was carried out as described in Materials and Methods. The RNA samples derived from BMDM treated with various agonists or from RAW264 cells are the same as those shown in Fig. 5. Shown are the protected bands separated on an 8% polyacrylamide sequencing gel, with the position of the undigested 420-base probe (run on a separate lane on the gel) indicated by the arrow. The size of each protected band was ascertained by comparison with a sequencing ladder run in parallel. Under precisely the same conditions, RNA from MOPC.31C, L929, or Lewis lung carcinoma cells failed to protect the probe from digestion (not shown). (B) Detection of short c-fms transcripts by RNA dot blot hybridization. Total RNA (10 μg) from the cell lines indicated was dissolved in 50% formamide-8% formaldehyde-1× SSC (0.15 M NaCl plus 0.015 M sodium citrate), heated to 68°C for 15 min, transferred to Hybond N (Amersham) membranes under vacuum, washed 10× SSC, and then baked overnight at 80°C. The treatments of the BMDM and RAW264 cells are the same as in Fig. 3 and 5. Duplicate blots were hybridized with either 32P-labeled antisense or sense RNA homologous to the 5' end of c-fms to cDNA bp 186 protected as for RNase protection (see Materials and Methods). The hybridization was carried out in 50% formamide-5× SSC-5× Denhardt's solution-0.1% SDS at 42°C. The blots were washed in 0.1× SSC-0.1% SDS at 65°C and then scanned with an AMBIS Radioanalyser. The radioactivity (counts per minute) present in each dot was counted, and the ratios are presented as the counts bound with the antisense probe/counts bound with the sense probe (corrected for the length of the probe).
FIG. 7. (a) Transient transfection analysis of the activity of the c-fms promoter, using CAT reporter gene constructs. Cells were transfected with 10 µg of reporter plasmid by electroporation (see Materials and Methods), and CAT expression was assayed after 48 h. The map of the reporter constructs is shown in panel b, and the restriction map of the 7-kb *ApaI* genomic DNA fragment encompassing the 5' end of the murine c-fms gene is shown in panel c. Restriction enzyme sites: A, *ApaI*; S, *SacI*; P, *PvuII*; H, *HaeIII*; X, *XbaI*; Xh, *XhoI*; Ev, *EcoRV*. In panel a, RAW264 cells were transfected with each of the constructs; then 10⁻⁷ M PMA was added to half the cells (shaded bars), while the remainder were untreated (solid bars). The results are averages of two separate experiments, each of which involved two separate transfections and separate preparations of plasmid.
restriction sites in the multiple cloning site, pGL-Basic provided a much lower negative control than did pCAT-Basic. Figure 9A compares the activities of three constructs, a 0.3-kb c-fms promoter, a 3.5-kb c-fms promoter, and a minigene construct (pGL6.7fms) containing the 3.5-kb 5’ flanking sequence plus the whole of intron 2 and the beginning of exon 3. The results obtained with the first two constructs confirm those obtained with the CAT vectors; the activity of the c-fms promoter relative to the β-actin promoter did not clearly distinguish macrophages from nonmacrophages. The inclusion of the intron 2 sequences reduced reporter gene expression in RAW264 cells but almost completely abolished activity in MOPC.31C, L929, and Lewis lung carcinoma cells. Also shown in Fig. 9A is the comparative activity of the SV40 early promoter and enhancer in the positive control vector pGL-Control. The β-actin promoter was slightly less active in MOPC.31C cells than in RAW264 cells and less again than in L929 and Lewis lung carcinoma cells. We have confirmed that the levels of β-actin mRNA and the rate of transcription of the β-actin gene in run-on transcription assays are similar in each cell population (not shown), so the expression of this construct provides some indication of transfection efficiency in each case. By contrast, pGL-Control (the SV40 early promoter) was 20- to 50-fold more active in MOPC.31C cells than in RAW264 cells, and in L929 and Lewis lung carcinoma cells, it was a further 10-fold less active. These data suggest that the SV40 enhancer contains tissue-specific cis-acting elements.

The results of the primer extension experiments in Fig. 3
and 5 suggest that CSF-1, LPS, and PMA reduce c-fms mRNA levels in macrophages by blocking transcription elongation within intron 2. Figure 9B shows the effects of the three agonists on c-fms promoter activity, using the luciferase reporter constructs above. CSF-1 had no effect in transient transfections with any of the reporter plasmids, which may reflect an artifact of transient transfection. In another study, we showed that the introduction of DNA into macrophages prevents them from responding to CSF-1 with increased growth (28) and prevents induction of the CSF-1-responsive urokinase plasminogen activator promoter (27a). By contrast to the data obtained with the CAT reporter constructs, PMA exerted a slight inhibition of the activity of the longer (3.5-kb) c-fms promoter but had a far more marked effect on the activity of the construct containing intron 2. LPS was more selective; it caused no inhibition of the activity of the 3.5- or 0.3-kb c-fms promoter but reduced the activity of the intron-containing construct three- to fivefold. The effects of the two agents were selective. In transfections parallel to those shown in Fig. 9, the SV40 promoter in pGL-Control was actually trans activated by LPS and PMA, whereas the β-actin promoter was relatively unaffected (not shown).

DISCUSSION

The c-fms gene in humans is closely linked to the gene encoding the platelet-derived growth factor (PDGF) receptor on chromosome 5 (20). Exon 1, located less than 500 bp downstream of the PDGF receptor gene, is transcribed only in trophoblasts and choriocarcinomas (20, 30). In human monocyte cell lines, transcription initiates immediately 5' of exon 2, which contains the initiation codon. Figures 1 and 2 show that the sequences of the mouse and human c-fms genes adjacent to exon 2 are conserved and that murine primary macrophages and the macrophage line RAW264 produce transcripts initiated at a cluster of transcription start sites in this region. These data are consistent with analysis of the human c-fms gene in macrophages (20), which identified a similar diversity of transcription start sites. The presence of multiple clustered transcription starts is common in TATA-less promoters (1, 26). The other element typically involved in specifying accurate transcription initiation is the CCAAT box, which is usually placed 50 to 90 bp upstream of the transcription start site (6). The repeated CCAAT-like motif around 60 to 100 bp from the multiple transcription start sites of c-fms, although it does not fit the CCAAT box consensus sequences, does bind multiple DNA-binding proteins in all cell lines tested, including at least one nuclear protein that also binds the CCAAT box sequence of the ubiquitously active H2-K promoter (unpublished data).

Taken together, the transient transfection and reverse transcriptase primer extension analyses in Fig. 2, 3, 5, 7, 8, and 9 suggest that expression of full-length c-fms mRNA in macrophages is controlled primarily by sequences in intron 2 that probably mediate tissue-specific transcription elongation. This conclusion contrasts with the claim that the homologous human c-fms promoter directs monocyte-specific expression of a CAT reporter gene in transient transfection analysis (20). Given the substantial homology between the promoters from the two species (Fig. 1) and the conserved patterns of expression, it seems unlikely that this
who suggested that the gene is controlled via differential regulation of tissue specificity as well as detectability. This possibility is
comparable abundance to using a full-length cDNA, we could not detect transcripts in L929 or MOPC.31C cells, whereas they were of
completely exclude this as an explanation for tissue specificity. In nuclear run-on transcription assays
phages with exon 2 primers (A and B) and the intronic primer
transcript synthesis rather than mRNA stability. Hence, we
a murine macrophage line by LPS occurred at the level of
intron.
the attenuated transcripts end in the case of
macrophages, they
the primer extension data (Fig. 6B)
hybridization (Fig. 6B)
 heterogeneous, part
heterogeneous and stem-loops in murine macrophages treated with LPS, PMA, and CSF-1.
Control of gene expression by at the level transcription
elongation has been observed in many cellular genes, including the adenosine deaminase, c-myc, c-myb, c-fos, and epidermal growth factor receptor (EGF-R) genes (see references 4, 7, 12, 19, and 27 and references therein). The EGF-R gene is particularly interesting since it is another member of the protein tyrosine kinase family of receptors and directs tissue-specific differentiation of a number of epithelial cell lineages. The EGF-R gene, like c-fms, is expressed from a TATA-less promoter and initiates from multiple clustered sites (12). Promoter activity does not correlate with patterns of expression, and a major determinant of full-length mRNA production is a transcription termination site in the first intron. As with this and most other examples of transcription attenuation, we have not been able to define exactly where the attenuated transcripts end in the case of c-fms. Although the primer extension data (Fig. 2 and 3) and dot blot hybridization (Fig. 6B) indicate that short transcripts are as
abundant in nonmacrophages as is the full-length mRNA in macrophages, they could not be detected by Northern blotting with a cDNA or a genomic DNA probe (not shown), and no bands protected from S1 nuclease were detected in nonmacrophages in the published studies of the human c-fms gene (20, 30). Similarly, we could not detect the short transcripts in nonmacrophages by RNase protection (Fig. 6A). In every case in which the 3′ ends of attenuated transcripts have been identified, they are extremely heterogeneous (27) even when analyzed in cell-free transcription assays (16). Since the 5′ ends of the c-fms transcripts are also heterogeneous, part of the reason they cannot be detected in Northern blots or nuclease protection may be that they do not form a definable band on a gel. An additional problem influencing detection of the short transcripts by nuclease protection is the abundance of extended GC-rich inverted repeats in exon 2 and the beginning of intron 2. In fact, the mouse sequence from ∼70 to +110 contains 63% GC residues. GC-rich stem-loops in both DNA and RNA are remarkably stable (13). Furthermore, there is the potential for interchain hybridization between short c-fms transcripts because of the inverted repeats. The existence of duplexes and stem-loops in short c-fms transcripts would probably reduce hybridization in nuclease protection assays, and any regions of mismatch or loop formation would not be protected from cleavage.

Those attenuators that have been characterized in prokaryotic and eukaryotic systems often contain GC-rich motifs with dyad symmetry (27). The c-fms sequences in exon 2, early in intron 2 (Fig. 4), and in exon 3 (mouse cDNA bp 127 to 139; 5′-CAGGGGCCCCGCTG-3′) clearly resemble other attenuator sequences, including those of the c-fos gene (5′-TCCCCGGCGCGGGA-3′) (18), the c-myc gene (5′-GCCCTGTTGGGC-3′) (19), and the TAR region of human immunodeficiency virus type 1 (as shown in Fig. 4). Homologies between c-fms sequences and the c-fos and c-myc terminators may be of functional significance. The c-fos attenuator is apparently involved in the regulation of c-fos transcription elongation in macrophages (7). c-myc is expressed in myeloid progenitor cells, and transcriptional elongation is down-regulated during myeloid differentiation (19). There might conceivably be a link between the control of transcription elongation of c-myc and c-fms.

In summary, we have provided evidence that tissue-specific expression of the murine c-fms gene in macrophages and its regulation by specific agonists involve sequences in intron 2 that probably control transcription elongation. Because c-fms is the receptor for the major macrophage growth and differentiation factor CSF-1, selective elongation of c-fms transcripts may be viewed as the rate-limiting event in macrophage differentiation.

REFERENCES