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Regulation of Urokinase-Type Plasminogen Activator Gene Transcription by Macrophage Colony-Stimulating Factor

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The mouse urokinase-type plasminogen activator (uPA) gene was used as a model macrophage colony-stimulating factor (CSF-1)-inducible gene to investigate CSF-1 signalling pathways. Nuclear run-on analysis showed that induction of uPA mRNA by CSF-1 and phorbol myristate acetate (PMA) was at the transcriptional level in bone marrow-derived macrophages. CSF-1 and PMA synergized strongly in the induction of uPA mRNA, showing that at least some components of CSF-1 action are mediated independently of protein kinase C. Promoter targets of CSF-1 signalling were investigated with NIH 3T3 cells expressing the human CSF-1 receptor (c-fms). UPA mRNA was induced in these cells by treatment with CSF-1, and a PEA3/AP-1 element at −2.4 kb in the uPA promoter was involved in this response. Ets transcription factors can act through PEA3 sequences, and the involvement of Ets factors in the induction of uPA was confirmed by use of a dominant negative Ets-2 factor. Expression of the DNA binding domain of Ets-2 fused to the lacZ gene product prevented CSF-1-mediated induction of uPA mRNA in NIH 3T3 cells expressing the CSF-1 receptor. Examination of ets-2 mRNA expression in macrophages showed that it was also induced synergistically by CSF-1 and PMA. In the macrophage cell line RAW264, the uPA PEA3/AP-1 element mediated a response to both PMA and cotransfected Ets-2. uPA promoter constructs were induced 60- to 130-fold by Ets-2 expression, and the recombinant Ets-2 DNA binding domain was able to bind to the uPA PEA3/AP-1 element. This work is consistent with a proposed pathway for CSF-1 signalling involving sequential activation of fms, ras, and Ets factors.

Macrophage colony-stimulating factor (CSF-1) acts primarily on cells of the mononuclear phagocyte lineage to control growth, differentiation, and survival of precursors as well as functional modulation of mature macrophages (59). The cell surface receptor for CSF-1 (CSF-1R) is a tyrosine kinase, the product of the c-fms proto-oncogene (reviewed in references 59 and 68), first identified by its homology to the viral oncogene v-fms from the McDonough feline sarcoma virus (69).

Binding of CSF-1 is required for the kinase activity of the normal CSF-1R, whereas the v-fms product is constitutively active. The CSF-1R is most closely related to the platelet-derived growth factor receptor and the c-kit gene product (59).

When CSF-1 binds to its receptor, a range of cellular proteins and the receptor itself are phosphorylated (22). Like the platelet-derived growth factor receptor, CSF-1R binds and activates the 85-kDa subunit of phosphatidylinositol 3’ kinase (55), but the role of phosphatidylinositol 3’ kinase in signal transduction is not known. Other proteins which become tyrosine phosphorylated in response to CSF-1 include src family kinases (17), tyrosine phosphatase PTP-1C (84), Shc proteins, and an uncharacterized 150-kDa protein potentially involved in the ras signalling pathway (45), although these may not all be direct substrates of the receptor. The ras GT-Pase-activating protein (GAP)-associated proteins p62 and p190, and to a small extent GAP itself, have been reported to be phosphorylated in response to activated CSF-1R in fibroblasts (31, 55), but this has not been observed with macrophages (55).

Some of the actions of CSF-1 are mimicked by the protein kinase C (PKC) agonist phorbol myristate acetate (PMA) (75), leading to speculation about the role of PKC in CSF-1 signalling. Evidence for an increase in production of diacylglycerol (an activator of PKC) after CSF-1 treatment of monocytes and bone marrow-derived macrophages (BMM) (34, 76) and for an increase in membrane-bound PKC activity (34, 66) has been presented, but recent experiments fail to support these findings (35). The generation of diacylglycerol is commonly the result of activation of phospholipase C-γ, an enzyme which cleaves phosphatidylinositol-4,5-bisphosphate to diacylglycerol and inositol-1,4,5-trisphosphate. Unlike the platelet-derived growth factor receptor, CSF-1R does not activate phospholipase C-γ (21), and there is no associated increase in phosphatidylinositol turnover (34, 81). An alternative route for diacylglycerol production may be by activation of a phosphatidylcholine-specific phospholipase C (83).

Evidence from a number of groups points to CSF-1-activated mitogenic signal pathways involving activation of fms, ras, raf, Ets, and myc factors (7, 9, 39, 60). This may not be a simple linear pathway, as recent experiments with a macrophage cell line showed that activation of Raf-1 was independent of ras (10). The involvement of p21 ras is well established in experiments with fibroblasts. CSF-1 treatment of c-fms-expressing NIH 3T3 cells and BAC1.2F5 macrophages increased the amount of active GTP-bound p21 ras (10, 26, 31). Microinjection of anti-p21 ras antibodies into v-fms-transformed fibroblasts caused reversion of the transformed phenotype (70), as did overexpression of the catalytic domain of GAP (7) and expression of a dominant negative Ets protein (39). Raf-1 was activated by CSF-1 treatment, and expression of oncogenic v-raf in a macrophage cell line led to CSF-1-independent growth and constitutive expression of ets-2 and c-myc mRNAs (9). Mutation of the CSF-1R at tyrosine 809, an autophosphor-
ylation site, prevented the mitogenic response to CSF-1 and impaired induction of c-myc; while induction of c-fos and junB was unaffected (60, 64). The mitogenic response could be restored by either c-myc (60), ets-2 (39), or ets-1 (61) expression.

Progress in defining promoter targets of CSF-1 action has been made in studies of the long terminal repeat of NVL3, a member of the VL30 family of mouse retrotransposons. A ras-responsive promoter element which also mediated a response to CSF-1 in NIH 3T3 fibroblasts expressing c-fms was found (7). The sequence element is a compound PEA3/AP-1-like site (54). Additional evidence for the role of p21ras in the CSF-1 signalling pathway was obtained by showing that the catalytic domain of GAP inhibited c-fms-mediated activation of this element (7). Expression of a dominant negative Ets protein (Ets-2 DNA binding domain–LacZ fusion protein), which presumably binds the PEA3 sequence, also prevented CSF-1 activation of this element (39). This work provided good evidence for the roles of p21ras and Ets factors in both CSF-1-mediated mitogenesis and induction of the NVL3 gene. More recent data have implicated Ets factors in c-myc induction by CSF-1 (61), in regulation of the CSF-1-responsive macrophage scavenger receptor gene (82), and in CSF-1-mediated induction of the c-fos gene (33).

The ultimate aim of studies of growth factor-activated signal transduction is to find all the molecular links from lipid binding at the cell surface to transcription factor activation and gene induction. Studies of nuclear targets of CSF-1 action in macrophages have been hampered by a lack of well-characterized genes which are induced by CSF-1 in transfecible cell lines, as primary macrophages and macrophage-like cell lines are difficult to transfect efficiently (73). This paper addresses the regulation of urokinase-type plasminogen activator (uPA), which is acutely regulated by CSF-1 in mature macrophages.

uPA is a serine protease which cleaves the proenzyme plasminogen to plasmin, a protease of broad specificity (reviewed in references 18 and 52). Plasmin directly catalyzes the degradation of the extracellular matrix and also acts indirectly by activating other proenzymes such as procollagenase. uPA activity is associated with processes which require tissue remodeling or cell movement, such as wound healing and involution of the mammary glands, and is frequently expressed at the invading front of cancerous tissue. Since macrophage function requires migration through tissues, uPA may be required to allow the necessary extracellular matrix degradation. uPA activity was first shown to be induced by CSF-1 in bone marrow-derived macrophages (BMM) (42). Accumulation of uPA mRNA in response to CSF-1 has been confirmed to occur in BMM (30) and in the macrophage-like cell line RAW264 (72). The level of macrophage or monocyte uPA mRNA has also been found to be increased by PMA (30) and gamma interferon (15) and decreased by dexamethasone and cyclic AMP (cAMP) agonists such as cholera toxin (15).

In this work we have examined the regulation of the uPA gene by CSF-1 and PMA. These agents synergistically induced uPA mRNA in macrophages, suggesting divergent pathways of activation. The difficulty of transfecting primary macrophages has meant that other cell types have had to be used for transient-transfection analysis. A PEA3/AP-1-like element at -2430 of the mouse uPA promoter was shown to be important in the induction of the gene by PMA in RAW264 cells and by CSF-1 in NIH 3T3 cells expressing c-fms. This element was first described by Rorth et al. (56) as being required for induction of uPA transcription by PMA and epidermal growth factor in keratinocytes. The element is conserved in mouse, pig, and human promoters (12) and is required for induction by PMA in a pig kidney epithelial cell line (41) and by PMA and basic fibroblast growth factor in NIH 3T3 cells (5). Hence, diverse signalling pathways can converge on this one element. Here we present evidence that Ets-2 can bind and activate transcription through this element.

MATERIALS AND METHODS

Materials. Recombinant human CSF-1 was kindly provided by Chiron Corp. pPacR (which was obtained from Signals). Cell culture. BALB/c BMU were differentiated in culture with CSF-1 as previously described (73). The RAW264 macrophage-like cell line (53) is derived from tumor cells of an Abelson leukemia virus-infected mouse. This cell line was transformed in bacteriological petri dishes in RPMI 1640 10% fetal calf serum. NIH 3T3 fibroblasts, NIH 3T3 cells expressing human c-fms (63), and NIH 3T3 cells expressing both c-fms and the human Ets-2 DNA binding region fused to luc (39) were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum.

Northern (RNA) analysis. Total RNA was extracted from cells by the method of Chomczynski and Sacchi (13). Total RNA (10 μg) was run on MOPS (morpholinepropane sulfonic acid)–formaldehyde denaturing gels, transferred to Hybond N nylon membranes, and hybridized with 32P-labelled DNA probes. The probes were used mouse uPA, a 1.2-kb PstI cDNA fragment (4); mouse Ets-2, excised from the plasmid PstEPE (obtained from R. Maki, La Jolla Cancer Research Foundation); IIS rRNA, a 24-bp oligonucleotide complementary to sequence 18S rRNA (5 CAT GCC AAC GAC CAT CAC T3) and human K-ras (rap1A), a gene fragment obtained by PCR.

Nuclear run-on analysis. The plasmids were used pGEM2 (Promega) (as a control); pU5', a genomic fragment of mouse uPA from +8 to +398 (including exon 1, intron 1, and the beginning of exon 2) in pBlueScript; pUAP', a fragment encompassing bp 424 to 1643 of the 2.3-kb mouse uPA cDNA in pGEM2; β-actin, a 1.8-kb chicken β-actin cDNA fragment in pGEMZ; c-fms, p575 containing the 3.7-kb mouse c-fms CDNA, obtained from L. Rohrschneider (Fred Hutchinson Cancer Research Center, Seattle, Wash.); lysozyme, pMILC, which contains a 780-bp fragment of mouse lysozyme M cDNA, obtained from R. Renkawitz (Max Planck Institute, Munich, Germany).

Plasmid was linearized, phenol-chloroform extracted, ethanol precipitated, and resuspended in 10 mM Tris-HCl (pH 8)–1 mM EDTA per 1 μl of plasmid (μg). pGEMZ 2.3-kb fragment (10 μg) was slot blotted onto Hybond N membranes. Cells (2 × 10^6 to 3 × 10^6 per treatment group) were harvested and rinsed twice with ice-cold phosphate-buffered saline (PBS). The cells were lysed with 5 ml of ice-cold lysis buffer (10 mM Tris HCl [pH 8.0], 10 mM NaCl, 3 mM MgCl2, 0.5% Nonidet P-40), pelleted (500 × g, 5 min), and then resuspended in 1 ml of lysis buffer. Nuclei were pelleted (500 × g, 5 min) and resuspended in 100 μl of glycerol storage buffer (50 mM Tris HCl [pH 8.3], 40% glycerol, 5 mM MgCl2) with 40 U of RNasin (Boehringer Mannheim). To 100 μl of nuclei were added 100 μl of reaction buffer (10 mM Tris HCl [pH 8.0], 5 mM MgCl2, 0.3 M KCl, 1 mM ATP, 1 mM GTP, 5 mM dithiothreitol) and 50 μl of [α-32P]UTP (3,000 Ci/mmol; Amersham). Nuclei were incubated at 30°C for 30 min with shaking. RNA was then extracted by the method of Chomczynski and Sacchi (13) with the addition of 20 μg of carrier yeast tRNA (Boehringer Mannheim).

Filters were prehybridized and hybridized in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH2PO4, and 1 mM EDTA [pH 7.7]), 5× Denhardt’s solution, 0.5% salmon dextran sulfate (pH 7.0), 50 μg of denatured, sheared calf thymus DNA/ml, and 200 μg of yeast RNA per ml at 42°C in small glass scintillation vials inside rolling hybridization bottles. The hybridization solution was removed after at least 6 h of incubation, and equivalent counts of RNA from each sample in 0.5 ml of hybridization solution were added. Hybridization proceeded for 36 h. The filters were washed twice for 10 min each time with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 42°C, twice for 20 min each time with 1× SSC-0.1% SDS at 55°C, and once for 30 min with 2× SSC with 10 μg of RNase A at 37°C and finally were rinsed several times in 2× SSC. The filters were exposed for 2 days to 2 weeks.

Transfection analysis. RAW264 cells were transfected by electroporation with 10 μg of plasmid DNA as described previously (73). We found that the voltage needed to be optimized for the individual electroporator in use, as delivered voltages differed from the readout values. puPA/CAT2 plasmids containing the mouse uPA promoter linked to the chloramphenicol acetyltransferase (CAT) gene were constructed (Fig. 3E) and have been described (39, 56). Plasmid pPR99, constructed by Rorth et al. (56), contains a 90-bp HaeIII fragment of the mouse uPA promoter (−2446 to −2356) linked to the uPA proximal promoter (−114 to +398) and the Escherichia coli CAT gene. The related plasmids pPPI19 and pPR127 contain point mutations in the PEA3/AP-1 site of the HaeIII fragment (56) (see Fig. 3E). pSPI26 contains only the proximal promoter (−114 to +398) and the CAT gene. Plasmid pGluPA-6.6 contains the same promoter region (−6616 to +398) as puPA/CAT2, inserted into the HindIII site of the luciferase reporter plasmid pGL2 (Promega). Plasmid pGLuc90 is equivalent to pPR99 but drives the luciferase reporter gene. The mouse Ets-2 expression vector EtsPEPE and PUPECE were obtained from R. Maki (La Jolla Cancer Research Foundation). Mouse E11 expression vector was obtained from Martin Reussel (St. Jude Children’s Research Hospital, Memphis, Tenn.). Mouse PEA3 CDNA (a gift from J. Hassell, McMaster University, Hamilton, Ontario).
Northern analysis of uPA and ets-2 mRNA expression in RAW264 cells and BMM. RAW264 cells and 6-day-old BMM were plated overnight (16 h) with or without 10^3 U of CSF-1 per ml. Cells were then either harvested or treated for a further 4 h with 200 ng of PMA per ml. Cell treatments were as follows: control, 16 h without CSF-1; + CSF-1, 16 h with CSF-1; + PMA, PMA added to medium for 4 h after incubation without CSF-1 for 16 h; and + CSF-1 + PMA, PMA added to medium for 4 h after incubation with CSF-1 for 16 h. Total RNA was extracted and hybridized with cDNA probes for uPA and Ets-2. An oligonucleotide probe for 18S rRNA is shown as a control for loading.

FIG. 1. Northern analysis of uPA and ets-2 mRNA expression in RAW264 cells and BMM. RAW264 cells and 6-day-old BMM were plated overnight (16 h) with or without 10^3 U of CSF-1 per ml. Cells were then either harvested or treated for a further 4 h with 200 ng of PMA per ml. Cell treatments were as follows: control, 16 h without CSF-1; + CSF-1, 16 h with CSF-1; + PMA, PMA added to medium for 4 h after incubation without CSF-1 for 16 h; and + CSF-1 + PMA, PMA added to medium for 4 h after incubation with CSF-1 for 16 h. Total RNA was extracted and hybridized with cDNA probes for uPA and Ets-2. An oligonucleotide probe for 18S rRNA is shown as a control for loading.

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Electrophoretic mobility shift analysis (EMSA). Extracts were prepared from approximately 3 × 10^6 BMM nuclei by a modification of the method of Osborn et al. (48). Cells were harvested, washed with PBS, and resuspended in 5 ml of hypotonic wash buffer (HWB) (10 mM HEPES [N-2-hydroxyethylpiperazine-N’-2-ethanulsuronic acid] pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA (ethylene glycol-bis [β-aminoethyl ether]-N,N,N’-N’-tetraacetic acid). After the extracts were pelleted and resuspended in 0.5 ml of HWB, 1 ml of HWB containing 0.05% Nonidet P-40 was added. Nuclei were spun down (500 × g, 5 min, 4°C; Heraeus Biofuge) and resuspended in 50 to 100 μl of nuclear extract buffer (20 mM HEPES [pH 7.9], 0.42 M NaCl, 20% glycerol, 0.2 mM EDTA, 0.2 mM EGTA) and left on ice for 10 min. Nuclei were spun down (15,000 × g, 10 min, 4°C; Heraeus Biofuge) and the supernatant was removed and stored at −20°C. All solutions were ice cold and contained 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiotreitol, 10 μg of leupeptin per ml, and 10 μg of aprotinin per ml. Protein extract (1 to 2 μg in 2 μl of nuclear extraction buffer) was incubated with 0.027 pmol of end-labelled double-stranded oligonucleotide probe in a total volume of 10 μl containing 20 mM HEPES (pH 7.9), 0.5 mM dithiotreitol, 12% glycerol, 50 mM NaCl, 0.5 μg of poly[d(I-C)], and 1 mM EDTA. After the addition of protein, samples were incubated at room temperature for 30 min and run on 5 to 6% acrylamide gels (acrylamide-to-bis ratio, 29:1) at 100 V in 0.5× Tris-borate-EDTA buffer. The gels were dried and visualized by autoradiography.

Mouse Ets-2 DNA binding domain (amino acids 334 to 466) was expressed in E. coli as a glutathione S-transferase (GST) fusion protein with the vector pGEX2T (Pharmacia). Protein was purified with glutathione-agarose (Sigma), and 10 ng of protein was used in EMSA as described above, with the omission of poly[d(I-C)] from the binding reaction. Expressed and purified GST protein was used as a control.

RESULTS

Northern analysis of uPA in macrophages. The effects of CSF-1 and PMA on uPA mRNA levels were examined with BMM and the CSF-1-independent macrophage-like cell line RAW264 (Fig. 1). Cells were plated overnight either with or without CSF-1 and then either harvested or treated for a further 4 h with PMA. The level of uPA mRNA was approximately 40-fold higher in CSF-1-treated BMM than in CSF-1-starved cells. The decline in uPA mRNA level with overnight CSF-1 starvation is fully reversible with the readdition of CSF-1 (71), so the decrease in mRNA level is not due to cell death. RAW264 cells also responded to CSF-1, giving an induced level of uPA mRNA that was approximately 25% of that in CSF-1-treated BMM. In six experiments, RAW264 cells responded to CSF-1 with three- to sixfold inductions of uPA mRNA during 8- to 24-h incubations (71, 72). In experiments examining the time course of induction of uPA mRNA by CSF-1 in both BMM and RAW264 cells, mRNA levels were increased after 1 to 2 h, reached a maximum at 8 to 16 h, and remained elevated above basal levels thereafter, provided that CSF-1 concentrations remained saturating (25, 30, 72).

The tumor-promoting phorbol ester PMA is believed to exert most of its effects by activation of PKC. PMA alone had a moderate effect on uPA levels in BMM but gave a very large increase in uPA levels in RAW264 cells (Fig. 1). It has been suggested that similarities in the actions of CSF-1 and PMA in macrophages are due to a common activation of PKC (75). If PMA and CSF-1 both induce uPA by activation of PKC, then treatment with optimal concentrations of the two agents together should not have an additive effect. Adding PMA to CSF-1-pretreated BMM increased uPA mRNA levels 400-fold above the level for CSF-1-starved cells (Fig. 1). Since CSF-1 and PMA added alone gave 40- and 20-fold inductions, respectively, this effect is much greater than additive. The synergism of the two factors added at saturating concentrations indicates that CSF-1 does not induce uPA by merely activating PKC in a manner similar to that of PMA. Although CSF-1 and PMA may activate some of the same pathways, they do so in distinct manners such that synergy is possible. In RAW264 cells the effect of CSF-1 and PMA together was also more than additive. The effect was less striking than in BMM because of the large level of induction by PMA alone.

Ets family transcription factors are implicated in CSF-1 induction of the NVL3 and c-myc genes and in mitogenesis in fibroblasts (39, 61). Ets factors are a family defined by homology to the v-Ets oncogene product of E26 erythroblastosis virus and include the products of the ets-1, ets-2, erg, fli-1, spi-1 (PU.1), elf-1, and PEA3 genes (78). PU.1 is a macrophage- and B-cell-specific transcription factor (57), but PU.1 expression and interaction with its target sequence are not affected by CSF-1 (58). Ets-2 protein and mRNA were found to be induced in chicken BMM by the chicken myelomonocytic growth factor and PKC activators (8). In the mouse macrophage cell line BAC1.2F5, ets-2 mRNA was induced by CSF-1 (9). Examination of ets-2 mRNA expression in macrophages showed induction by PMA and CSF-1 closely paralleling that of uPA mRNA, with apparent synergism between the two stimuli (Fig. 1). A time course of the response to CSF-1 in BMM showed a peak of ets-2 mRNA expression at 1 to 2 h, preceding the major induction of uPA (25). In RAW264 cells, the response of ets-2 mRNA to CSF-1 was barely detectable even at 2 h (25), although the message was induced by PMA (Fig. 1). The possibility that transcription factor Ets-2 can regulate uPA expression is addressed later in this paper. If induction of Ets-2 is a prerequisite for uPA induction, then it would be anticipated that blockage of protein synthesis would prevent uPA induction. Investigations of such events are difficult, as both ets-2 mRNA and uPA mRNA are induced in macrophages by cycloheximide (14, 25).
were used. uPA 5' is a genomic clone containing the noncoding first exon, the first intron, and the beginning of exon 2. uPA 3' is a cdNA clone containing bp 424 to 1643 of the 2.3-kb mouse uPA cdNA. PGEM2 plasmid DNA was used as a control. (A) Six-day-old BMM were starved of CSF-1 for 15 h (control), grown in the continued presence of CSF-1 (+CSF-1), or starved of CSF-1 for 15 h prior to the addition of PMA for 1 h (+PMA) before the isolation of nuclei. (B) RAW 264 cells were grown without additions (control), with CSF-1 for 12 h (+CSF-1 12 hr), with CSF-1 for 4 h (+CSF-1 4 hr), or with PMA for 1 h (+PMA). Seven-day-old BMM grown in the continued presence of CSF-1 (+CSF-1) were assayed in the same experiment. These assays were performed with duplicate cell samples, and results are representative of two independent run-on analyses. CSF-1 was used at 104 U/ml, and PMA was used at 200 ng/ml.

Figure 2. Nuclear run-on analysis of transcription of the uPA, β-actin, c-fms, and lysozyme genes in BMM and RAW 264 cells. uPA 5' is a genomic fragment of uPA including exon 1 and intron 1. uPA 3' is a cdNA clone encompassing bp 424 to 1643 of the 2.3-kb mouse uPA cdNA. PGEM2 plasmid DNA was used as a control. (A) Six-day-old BMM were starved of CSF-1 for 15 h (control), grown in the continued presence of CSF-1 (+CSF-1), or starved of CSF-1 for 15 h prior to the addition of PMA for 1 h (+PMA) before the isolation of nuclei. (B) RAW 264 cells were grown without additions (control), with CSF-1 for 12 h (+CSF-1 12 hr), with CSF-1 for 4 h (+CSF-1 4 hr), or with PMA for 1 h (+PMA). Seven-day-old BMM grown in the continued presence of CSF-1 (+CSF-1) were assayed in the same experiment. These assays were performed with duplicate cell samples, and results are representative of two independent run-on analyses. CSF-1 was used at 104 U/ml, and PMA was used at 200 ng/ml.

The rates of uPA transcription in BMM and RAW 264 cells were compared by using equivalent counts per minute of run-on transcripts from the two cell types (Fig. 2B). Such an approach assumes that the total nascent transcripts produced per cell per unit of time is independent of the cell type. This is unlikely to be the case, as RAW 264 cells have been reported to proliferate more rapidly than BMM. However, even if it is assumed that RAW 264 nuclei incorporated twice as much radiolabel as BMM nuclei, the rate of transcription of uPA in BMM treated with CSF-1 was still apparently much greater than in RAW 264 cells with or without CSF-1 (Fig. 2B). The results of Northern and nuclear run-on analyses suggest that the CSF-1 responsiveness of the uPA gene is impaired in RAW 264 cells compared with that in BMM. CSF-1 and PMA treatments also affected the transcription of other genes studied. In both RAW 264 cells and BMM, transcription of c-fms and the lysozyme gene, which are macrophage marker genes, was reduced by CSF-1 and PMA. Both of these agonists are known to down-regulate c-fms mRNA in these cells (85).

Promoter studies in RAW 264 cells. In previous work, we showed that RAW 264 cells could be effectively transfected by electroporation but that BMM could not be transfected by the same method because of DNA-dependent cell death (73). Unfortunately no macrophage cell line which is readily transfec-
table and gives a good transcriptional response to CSF-1 has yet been identified. Previously reported analysis of the mouse uPA promoter in RAW 264 cells (12) showed that maximal expression required the first intron and 6.6 kb of promoter sequence. The full-length (6.6-kb) uPA promoter was induced sixfold by PMA, but no induction was seen with CSF-1 (72). Basal expression of the pig promoter transfected into RAW 264 cells was dependent on the PE3/AF-1 element first described by Rørth et al. (12, 56).

Rørth et al. (56) prepared plasmid pPR99 containing a 90-bp Hae III fragment with the PE3/AF-1 element (−2446 to −2356) linked to the uPA-proximal promoter region (−114 to +398) and the CAT reporter gene (Fig. 3E). The activity of this construct in RAW 264 cells was compared with that of the full-length promoter in puPACAT2 (−6.6 kb to +398) and that of the promoter truncated to −2657. Truncation of the promoter to −2657 reduced the basal level of expression (12) but gave little change in PMA inducibility (Fig. 3A). Thus, regions upstream of −2657 have enhancer activity (12) but are not required for the PMA response. The PMA-induced level of expression from pPR99 is seven to eight times that of the full-length promoter in puPACAT2. The higher level of inducibility may be due to the deletion of inhibitory regions, such as those found in the human promoter between −660 and −338 (11) and between −1824 and −1572 (77), both of which are 3' to the PE3/AF-1 element.

Mutating either the PE3 half (plasmid pPR119) or the AP-1 half (plasmid pPR127) (56) of the PE3/AF-1 site greatly reduced the PMA response (Fig. 3B). As the basal level of expression from these plasmids was very close to the experimental background level, it was not possible to accurately determine the fold induction by PMA. Using analogous constructs in a luciferase vector in which low-level activity is more easily detectable, we showed that the PE3/AF-1 and AP-1 mutations caused only a 50% drop in activity (25). Hence, the reductions in PMA-induced activity in pPR119 and pPR127 are not likely to be due to a drop in basal expression. The PE3/AF-1 element can mediate a response to PMA in RAW 264 cells, and both halves of the site are required for maximal induction. A plasmid with only the uPA-proximal promoter (−114 to +398), pPR126, was not PMA responsive (Fig. 3B).

Figure 1 shows that the expression of ets-2 mRNA closely paralleled that of uPA. The ets-2 and uPA genes may be regulated together through common pathways, or Ets-2 levels may control uPA mRNA levels. Since the peak of ets-2 mRNA expression precedes most of the uPA mRNA induction (25) and PE3/AF-1 sequences are bound by Ets family transcription factors (79), the second possibility is an attractive hypothesis. The relatively impaired response of RAW 264 cells to CSF-1 may be a consequence of the failure to induce Ets-2. For this reason, the effect of Ets-2 expression on promoter activity in
Figure 3C shows the results of the cotransfection of an Ets-2 expression plasmid (Ets.pECE) with pPR99 and mutated constructs into RAW264 cells. Ets-2 expression dramatically increased the activity of the uPA promoter in pPR99. Mutations of the PEA3 site (pPR119) and the AP-1 site (pPR127) both reduced the response to cotransfected Ets-2. It is likely that Ets-2 activates transcription directly through the PEA3/AP-1 site, but the possibility that Ets-2 induces another factor involved in transactivation cannot be ruled out. The fact that the AP-1 mutation decreases the
response to Ets-2 emphasizes the compound nature of this element; both halves of the element are required for full enhancer activity. The uPA proximal promoter region alone in pPRL26 did not respond to Ets-2. The residual Ets-2 response seen with the PEA3/AP-1 mutant constructs pPR119 and pPR127 may be due to another region within the 90-bp HaeIII fragment which has a potential Ets factor binding sequence (GGAT at −2396 bp in the mouse promoter). This sequence is not perfectly conserved among mouse, pig, and human promoters (12), and whether it has an enhancer function has not been established.

Transactivation of the uPA promoter is not a property of all Ets factors. Figure 3D shows results of cotransfection of uPA promoter constructs with expression vectors for the Ets family members Ets-2, Elf-1, PU.1, and PEA3. The promoter constructs used were pGLuPA-6.6, which contains the same promoter region as pPACAT2 (−6616 to +398) linked to the luciferase reporter gene, and pGL99, which is a luciferase version of pPR99 (Fig. 3E). For both plasmids, the induction by Ets-2 was 60- to 130-fold. It should be noted that the level of luciferase activity from pGL99 was approximately 25% of that from pGLuPA-6.6. Expression of PU.1 and Elf-1 had little or no effect on promoter activity, and PEA3 gave only a modest increase compared with that of Ets-2. Although native PU.1 is expressed relatively highly in RAW264 cells (57), we still observe some transactivation by coexpressed PU.1 on several other promoters. Our previous work showed that PU.1 from RAW264 nuclear extracts did not bind the uPA PEA3/AP-1 element under EMSA conditions (12). An Ets-1 expression vector gave the same magnitude of response as Ets-2 (result not shown). Ets-1 is not a good candidate for regulating expression of macrophages but not in macrophage or myeloid cell lines (37). Consistent with the results of nuclear run-on (Fig. 2), which show little effect of CSF-1 on uPA gene transcription in RAW264 cells, no activation of the uPA promoter by CSF-1 was detected in transfection analyses with this cell line (72). Stably integrated full-length (6.6-kb) promoter was also not responsive to CSF-1 (25). Results of transient and stable transfection therefore support the idea that the three- to sixfold induction of uPAmRNA in RAW264 cells (75) does not involve activation of the PEAS/AP-1 element and requires Ets action. The transient increases in uPAmRNA observed in NIH3T3/c-fms and the human Ets-2 DNA binding domain fused to the lacZ gene (pAPr-

![FIG. 4. Effects of CSF-1 and Ets-2 DNA binding domain on uPA mRNA expression in NIH 3T3 cells.](image)

4). This is similar to the result obtained for CSF-1 stimulation of NVL3 expression in NIH 3T3/c-fms. The NVL3 gene contains a PEA3/AP-1-like element which behaves as both a ras-responsive element and a CSF-1-responsive element (7). The possibility that the uPA PEA3/AP-1 element can behave as a CSF-1-responsive element was addressed by transient transfections into NIH3T3 cells.

**uPA promoter activity in NIH 3T3/c-fms.** Promoter elements involved in the CSF-1 response of NIH 3T3/c-fms were analyzed by transient transfection. Plasmid pPR99 and the mutant plasmids pPR119 and pPR127 (56) were transfected into NIH3T3 cells with and without cotransfected v-fms expression plasmid, which encodes a constitutively active form of the CSF-1R (62) (Fig. 5A). The basal level of activity of pPR99 was low in NIH3T3 cells, but v-fms expression activated this reporter by an average of 25-fold in three separate experiments. In contrast, the activities of reporters that contained point mutations in either the PEA3 site (pPR119) or the AP-1 site (pPR127) were not greatly stimulated by v-fms. In a second set of experiments, reporters were transfected into NIH3T3/c-fms cells and stimulation by CSF-1 was determined (Fig. 5B). In these experiments, pPR99 activity was stimulated three- to fourfold, while the activities of pPR119 and pPR127 were not significantly affected by the ligand-activated CSF-1R. The CSF-1-mediated increase in uPA mRNA (Fig. 4) is therefore likely to involve activation of the PEA3/AP-1 element and requires Ets family transcription factors.

**Proteins binding the PEA3/AP-1 element.** The transient transfection analyses described above implicate AP-1 or cAMP response element (CRE)-binding and Ets family transcription factors in the action of CSF-1 and PMA. EMSA was performed in order to determine whether AP-1 transcription factors are able to bind the octameric AP-1 site of the uPA element and whether any proteins binding the PEA3 site are detectable.

In EMSA the uPA PEA3/AP-1 element (uPA wt oligonucleotide) formed two major complexes with nuclear proteins from CSF-1-treated BMM (Fig. 6A). These complexes were abolished by mutation of the AP-1 half of the element (uPA
M5). Mutation of the PEA3 site (uPA M7) or use of a truncated oligonucleotide completely lacking the PEA3 site (uPA CRE) did not affect the formation of these complexes. Thus, the major proteins detectable under the conditions used bind only to the AP-1 half of the element. The possibility that phosphorylation may be necessary for factor binding to the PEA3 half was considered, but inclusion of phosphatase inhibitors in extraction and binding buffers did not alter the pattern of binding (25).

Incubation of CSF-1-treated BMM nuclear extract with an oligonucleotide able to bind AP-1 yielded one band (Fig. 6B). Cold uPA wt oligonucleotide was able to compete for proteins binding to the AP-1 site but was a little less efficient than self-competition with cold AP-1 oligonucleotide. Mutation of the AP-1 half of the uPA element (M8) abolished this competition, whereas mutation of the PEA3 half (M5) did not. Thus, the octameric site in the uPA PEA3/AP-1 element (TGAG GTCA) can bind some of the same factors which bind to a conventional AP-1 site (TGAGCTCA).

Treatment of BMM with CSF-1 or PMA increased the levels of proteins binding to the AP-1 oligonucleotide (Fig. 6C). With CSF-1 treatment, levels were highest at 1 h and subsequently declined. However, CSF-1 and PMA did not synergize to increase AP-1 binding activity. In fact, CSF-1 pretreatment prevented induction of AP-1 binding activity by PMA. The same pattern of inductions was found with labelled uPA wt oligonucleotide, although the degree of induction was less pronounced (result not shown). The low level of protein binding in extracts from cells treated with CSF-1 and PMA together indicates that uPA promoter activity does not correlate directly with the induction of AP-1 binding activity.

DNA binding of Ets-1 and Ets-2 is known to be impeded by a repressive domain under the conditions of EMSA (67). High-affinity binding may require either posttranslational modification or protein-protein interaction to relieve this repressive
effect. Perhaps because of this problem and the lack of good supershifting antibodies specific for Ets-2, to our knowledge native Ets-2 binding activity in nuclear extracts has not been clearly identified. Even using a consensus sequence determined for binding of recombinant Ets-2 DNA binding domain (44), we could not detect a PMA- or CSF-1-inducible protein in macrophages (result not shown), although ets-2 mRNA is clearly induced (Fig. 1). To examine whether Ets-2 can bind the uPA PEA3/AP-1 element, the DNA binding domain alone was expressed as a GST fusion protein. Figure 7A shows that the Ets-2 DNA binding domain binds the uPA wt oligonucleotide and can be competed for by the AP-1 mutant (uPA M5) and an Ets-2 consensus oligonucleotide but not by the PEA3 mutant (uPA M8). On the basis of competition with increasing concentrations of cold oligonucleotides, the uPA wt sequence was evidently a much lower-affinity site for Ets-2 binding than the Ets-2 consensus sequence (Fig. 7B).

**DISCUSSION**

A PEA3/AP-1 element was first identified in the polyoma virus enhancer. It mediated responses to serum, PMA, and the v-src, Ha-ras, v-mos, v-raf, and polyomavirus middle-T-antigen oncogenes (80) and has been termed a tetradeacanoyl phorbol acetate- and oncogene-responsive unit (28). Similar elements have been found in other genes such as collagenase (28), transforming growth factor β (50, 54), the NVL3 retrotransposon (54), keratin 18 (51), uPA (56), and the macrophage scavenger receptor (82) (Fig. 8). These elements have all been characterized as ras-responsive elements. Although Ratth et al. (56) mention that ras transformation of keratinocytes did not activate the uPA PEA3/AP-1 element, the element responded strongly to activated ras in NIH 3T3 cells (16) and RAW264 macrophages (25). The collagenase and keratin 18 PEA3 and AP-1 elements are more widely spaced (Fig. 8), but it is interesting that the insertion of 10 bp in both cases corresponds to one turn of the DNA helix and may give a similar orientation of binding factors.

As noted in the introduction, the uPA PEA3/AP-1 site at −2430 in the mouse promoter mediates induction by signals such as epidermal growth factor, PMA, and basic fibroblast growth factor. In this work we have found that the uPA PEA3/AP-1 element is involved in the transcriptional response to CSF-1 in NIH 3T3/c-fms. Both halves of the element were required for efficient activation of transcription by either v-fms or c-fms with CSF-1 (Fig. 5). This may reflect the fact that the PEA3 and AP-1 halves are comparatively weak binding sites for Ets and AP-1 proteins (Fig. 6 and 7). Induction of uPA mRNA by CSF-1 was prevented by both a dominant negative Ets protein (Fig. 4) and the catalytic domain of GAP (16). uPA induction by CSF-1 in this cell line therefore appears to involve ras signalling and activation of Ets transcription factors. This makes uPA regulation similar to that of both the NVL3 retrotransposon and c-myc (6, 7, 39, 61). Work on the induction of the NVL3 retrotransposon by CSF-1 in NIH 3T3/c-fms identified a PEA3/AP-1-like element mediating the CSF-1 response (7, 54). This element had been previously identified as a ras-responsive element (49). Another CSF-1-inducible gene, the macrophage scavenger receptor gene, contains a similar ras- and PMA-responsive element, although this element has not yet been directly shown to be activated by CSF-1 (82). Comparison of the NVL3, uPA, and scavenger receptor PEA3/AP-1 elements (Fig. 8) shows similar spacings of elements but a number of sequence differences. Wu et al. (82) found that Ets-2, but not Ets-1, was able to transactivate the scavenger receptor element and to cooperate with c-Jun. This differs from results for the uPA element in RAW264 cells, in which Ets-2 and Ets-1 have similar activities.
Introduction of a mutation at Tyr-809 in the CSF-1R prevents induction of c-myc, the NVL3 gene, and the mitogenic response in NIH 3T3/c-myctreated with CSF-1 (39, 60, 64). These effects are mimicked by the expression of dominant negative Ets-2 (39). CSF-1-induced mitogenesis can be restored to cells with the Tyr-809 mutation by the expression of ets-1, ets-2, or c-myc (39, 60, 61), and induction of c-myc appears to involve Ets factors (61). This implicates an Ets factor with binding specificity and activity similar to those of Ets-1 and Ets-2 in the CSF-1 signalling pathway in these cells. The identification of the NVL3 and uPA PEA3/AP-1 sequences as promoter targets of CSF-1 is an important step towards finding the specific transcription factors involved in signalling.

The 3' half of the uPA PEA3/AP-1 site is octameric and more closely resembles a CRE (TGACGTCA) than it does the consensus AP-1 site (TGACGTTC). The specificity between CRE and AP-1 sites is based on the following: that AP-1 factors can dimerize with the CRE-binding factors (CREB/ATFfamily) (29) and that AP-1 factors themselves can also bind to octameric sequences (1, 65). The wide range of Ets family proteins and AP-1 and CREB/ATF proteins makes the determination of which factors bind these elements a challenge. The octameric site in uPA is not responsive to cAMP and functions similarly to an AP-1 site (40). In extracts from pig kidney epithelial cells, c-Jun has been found to bind the pig uPA PEA3/AP-1 element (41). In another study, a c-Jun/ATF2 heterodimer has been shown to bind the AP-1 half of the uPA PEA3/AP-1 element (19). Macrophage AP-1-binding factors will also clearly bind to the octameric uPA AP-1 sequence (Fig. 6), although with slightly lower affinity than to a conventional AP-1 site. When either a labelled AP-1 oligonucleotide (Fig. 6C) or the uPA PEA3/AP-1 element (25) was used, treatment with CSF-1 or PMA increased levels of AP-1 factor binding. However, CSF-1 pretreatment prevented induction of AP-1 factors by PMA. Hence, the synergy seen in induction of uPA mRNA (Fig. 1) is not due to synergistic induction of general AP-1 binding activity.

The effect of PEA3 site mutation in transient-expression analysis (Fig. 3) showed that this element was functionally important. Despite this, with EMSA we were unable to detect any strong binding of macrophage nuclear factors to the PEA3 half of the uPA element. The two major detectable bands were both accounted for by proteins binding to the AP-1 site (Fig. 6). Conditions used in making nuclear protein extracts or in EMSA may be inadequate for detecting factors binding to the PEA3 site. In EMSA with extract from pig kidney LLC-PK1 (41). In HepG2 hepatoma cells, the cooperation between the PEA3/AP-1 site and a downstream AP-1 site was necessary for induction by PMA (47).

Although analysis of the CSF-1 response in transfected RAW264 cells was not possible, the PEA3/AP-1 element was identified as a PMA-responsive element in this cell line (Fig. 3B). The same element mediated a response to PMA in keratinocytes (56) and in the pig kidney epithelial cell line LLC-PK1 (41). In HepG2 hepatoma cells, the cooperation between the PEA3/AP-1 site and a downstream AP-1 site was necessary for induction by PMA (47).

Although both PMA and CSF-1 are able to activate the PEA3/AP-1 element, the evidence does not support the hypothesis that the pathways of activation are coincident. Both the synergy between CSF-1 and PMA in induction of uPA mRNA in BMM (Fig. 1) and the differential response of RAW264 cells to CSF-1 and PMA in transient transfections suggest that CSF-1 and PMA induce uPA by distinct mechanisms. PMA does not mimic the action of CSF-1. The two agonists may activate separate pathways or may have synergistic effects on the one signalling pathway. Although some evidence that CSF-1 activates PKC has been presented (34, 66), a recent study suggests that PKC activation is not significant, at least not in early signalling by CSF-1 (35). Like CSF-1 (2), PMA and PKC can activate Raf-1 (38). It will be interesting to
ascertain whether PMA can increase Raf-1 activation when added to CSF-1-treated cells.

PMA is frequently used in promoter studies without indication of its physiological relevance. There may be natural stimuli which act through PKC and strongly enhance the induction of uPA by CSF-1. Transforming growth factor β induces uPA in RAW264 cells and may act through a PKC-dependent mechanism (24). It is also possible that PMA provides a combination of stimuli to cells which does not resemble the action of any physiological molecule.

In this work we have provided evidence that the CSF-1 response of the uPA gene is distinct from the response to PMA, is mediated through a PEA3/AP-1 element, and requires activation of an Ets transcription factor, possibly Ets-2. Earlier evidence pointed to bifurcating signalling pathways from the CSF-1 receptor, one through ras, Raf, Ets, and myc factors and another leading to induction of fos and jun proteins (7, 9, 39, 60). Although ras is generally considered an activator of Raf-1, recent work showed that ras and Raf-1 were activated independently by CSF-1 in the macrophage cell line BAC1.2FS (10) and are therefore likely to be part of cooperating but separate pathways. Since expression of v-ras in BAC1.2FS macrophages leads to constitutive expression of c-myc in NIH3T3 cells which co-express the CSF-1 receptor and the c-myc in NIH3T3 cells which co-express the CSF-1 receptor and the catalytic domain of the GTPase-activating protein restores CSF-1 dependent mitogenic growth. Int. J. Oncol. 1:221–225.


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