Interaction between PU.1 and Another Ets Family Transcription Factor Promotes Macrophage-specific Basal Transcription Initiation*

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Numerous macrophage-restricted promoters lack TATA boxes or other conventional initiation motifs but contain high affinity binding sites (PU boxes) for the macrophage-restricted Ets family transcription factor PU.1. In RAW264 murine macrophages, multimerized PU boxes were not active as enhancers when placed upstream of a minimal promoter. To model their role in basal promoters, we inserted PU boxes into a promoterless luciferase reporter plasmid. Two sites, regardless of orientation, were necessary and sufficient to direct reporter gene expression in transient transfections of the RAW264 macrophage-like cell line. This activity was absent in transfected 3T3 fibroblasts but could be induced by PU.1 coexpression. Both the model promoter and the macrophage-specific mouse and human c-fms promoters were activated in RAW264 cells by other Ets family transcription factors, Ets-2 and Elf-1. In fibroblasts, the effects of PU.1 and Ets-2 were multiplicative, whereas overexpression of PU.1 in RAW264 cells reduced activation of c-fms or model promoters by the other Ets factors. The PU.1 and Ets-2 binding sites of the mouse c-fms promoter have been located by DNase footprinting. A conserved Ets-like motif at the transcription site, CAG-GAAC, that bound only weakly to PU.1, was identified as an additional critical basal c-fms promoter element. Comparison of studies on the model promoter, c-fms and other myeloid promoters provides evidence for a conserved mechanism that involves three separate and functionally distinct Ets-like motifs.

Members of the large Ets family of transcription factors are characterized by a DNA binding domain (usually in the C-terminal region) that is homologous to the viral oncogene v-ets (1) and recognizes purine-rich sequences typically with a 5'-GGAA-3' core. The most divergent member of the Ets family, PU.1, is known to be restricted to macrophages, B cells, mast cells, and neutrophils (2, 3) and is necessary for normal myelopoiesis (4–7). Apart from the Ets domain, PU.1 has a glutamine-rich activation domain and a proline-, serine-, threo-

nine-, and glutamic acid-rich (PEST) domain (8), which links the activation and Ets domains.

PU.1 recognition motifs have been shown to be important in the expression of many macrophage-restricted genes such as c-fms, which encodes the receptor for macrophage colony-stimulating factor (9, 10), CD11b (11), CD18 (12–14), FcyR1 (15, 16), FcyRIIIA (17), GM-CSF receptor (18), c-fes (19), the macrophage scavenger receptor (20), and the PU.1 gene itself (21, 22). Our own studies have concentrated upon the mouse and human c-fms promoters. Two functional PU.1 binding sites are present in the human c-fms promoter at −150 and −170 nucleotides from the start codon (−150H and −170H). Zhang et al. (10) provided evidence that the more distal site has higher PU.1 binding affinity, but deletion of either site lowers the basal promoter activity substantially (23), indicating that both are required. Only one of these PU.1 sites is clearly present in the mouse promoter (Fig. 1), but at −170M there is a candidate PU.1 site on the opposite strand.

The mouse and human c-fms genes and many of the macrophage-specific genes cited above lack proximal promoter elements that normally determine the site of initiation such as a TATA box, Inr (initiator) sequences, or the GC- rich sequences found in “housekeeping” genes (Fig. 1). The possibility that PU.1 might function in initiation in macrophage specific genes is favored by evidence that it can bind directly to both TFIIID and the retinoblastoma gene product (24) and the location of PU.1 sites 30–50 bp upstream of the multiple sites of transcription initiation in most of the promoters cited above. This paper contains an analysis comparing artificial promoters containing only PU.1 recognition sites with the macrophage-specific mouse and human c-fms proximal promoters. As an explanation for the presence of multiple purine-rich elements in macrophage-specific promoters, we propose that PU.1 needs to interact with another member(s) of the Ets transcription factor family to promote macrophage-specific transcription initiation. In the course of this study, we provide evidence for a separate role in initiation for GGAA motifs to which PU.1 binds weakly, if at all.

MATERIALS AND METHODS

Cell Culture—NIH3T3 (murine fibroblast line) and RAW264 (a murine macrophage-like line) were obtained from the American Type Culture Collection (Rockville, MD) and cultivated as described by Stacey et al. (25). HeLa cells were obtained from Dr. R. Sturm and cultured in Dulbecco’s modified Eagle’s medium plus 5% fetal bovine serum.

Nuclear Extracts—Nuclear extracts were prepared with a variation (9) of the method of Osborn et al. (26). Recombinant PU.1 (9) and GST-Ets-2 (25) were prepared and purified as described elsewhere.

The abbreviations used are: bp, base pair(s); CSF, colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; RLU, relative light units; CAT, chloramphenicol acetyltransferase.

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Transient DNA Transfection Analysis and Plasmid Construction—

The mouse pGL0.3fms, pGL3.5fms, and pGL6.7fms (27) and human 
-430fms and -150H mutant human -430fms (M1) (23) c-fms promot-
er-luciferase reporter plasmids are described elsewhere. Mutation of the 
-103 site of the mouse promoter from CAGGA to CACCAA mut-
-ant was performed in the pGL0.3fms plasmid according to the 
Quikchange™ methodology (Stratagene, La Jolla, CA) and confirmed 
directly by sequencing. The luciferase reporter plasmids pGL2C (SV40 
enhancer and promoter), the SV40 promoter plasmid pGL2P, and the 
basal plasmid pGL2B were obtained from Promega (Madison, WI). The 
mouse PU.1 expression plasmid PU-pECE, the mouse Ets-2 expression 
plasmid Ets2pECE, and the parent vector pECE used previously (9) 
were a gift from Dr. Richard Makil as were the pBL-CAT series plasmids 
(28). The Elf-1 expression vector, in which expression of the mouse 
protein is directed by the Rous sarcoma virus long terminal repeat, was 
a gift from Dr. Martine Roussel. The PU box model promoters were 
made by ligating the SV40 PU.1 box oligonucleotide into pGL2B, which 
had been cut with XhoI and end-filled. The double-stranded PU box 
oligonucleotide (5'-CTGAAAGAGGAACTTGGTTAGGTA-3') (28) was 
phosphorylated with T4 polynucleotide kinase prior to ligation to allow 
multimerization. Clones containing inserts were characterized for num-
ber and orientation of inserts by direct sequencing. Plasmids containing 
1–4 PU boxes in various orientations were isolated.

Plasmids were purified for transfection by the alkaline-SDS method 
(29) followed by affinity chromatography on a Qiagen column (Qiagen, 
Dusseldorf, Germany) or by CsCl density gradient centrifugation (29).

Cells were transfected with 10 μg of luciferase reporter constructs by 
electroporation (9). In co-transfections, 1 μg of each of the relevant 
expression plasmid or vector control was also added. CAT activities 
determined as described previously (30). Absolute activities on all 
figures are expressed as relative light units (RLU), or chloramphenicol 
acetyltransferase (CAT) activity, corrected for protein concentration. 
This RLU value is purely empirical and cannot be compared between 
figures. As in previous studies (9, 31), we have not used internal 
transfection controls because of concerns about their validity. Where 
controls are needed to assess relative promoter activity or the specificity 
of actions of co-transfected transcription factor expression plasmids, 
these are performed separately in parallel transfections.

RESULTS

DNA Binding Analysis—Recombinant PU.1 and GST-Ets-2 
was prepared as described above. To label the DNA strands, the 
pGL0.3fms reporter plasmid was cut with either HinIII (to 3' -label the 
upper strand) or MluI (to 3' -label the lower strand), and [3P]pCPDT 
was incorporated using the Klenow fragment of DNA polymerase. The 
plasmid was then re-cut with MluI or HinIII, respectively, to generate the 
appropriate labeled fragment. The probe fragment was purified by 
elution from a 5% TBE acrylamide gel. Binding reactions were constitu-
ted as follows; to 25 μl of bufer D was added 2 μl of poly(dI-dC) (1 
mg/ml) and nuclear extract (1, 5, or 10 μg), recombinant GST-Ets-2 (1, 
5, or 10 μl), or recombinant PU.1 (1, 5, or 10 μl). Probe (2 μl, approxi-
mately 108 cpm) was added, and the volume was adjusted to 50 μl/ 
reaction with MilliQ water. Binding reactions were left at 25 °C for 60 
min before adding 51 μl of DNsse 1 (50 μg/ml in 10 mM MgCl2, 5 mM 
CaCl2). After 30 s, 100 μl of stop solution (30 mM EDTA, 200 mM NaCl, 
1% SDS, 100 μg/ml tRNA) was added, followed by 30 μl of ammonium 
acetate solution (3 mM ammonium acetate, 20 mM EDTA). DNA was 
precipitated with 2 volumes of ethanol, washed, dried, and redissolved 
in 5 μl of sequencing gel loading buffer. Aliquots (2 μl) of each reaction 
were analyzed on a sequencing gel, using a c-fms sequencing ladder 
(0.3fms, pGL2B primer) for calibration.

RESULTS

A PU box Multimer Does Not Function as an Enhancer in 
RAW264 Cells—Some previous studies of PU.1 (8, 28) have 
presented the view that PU.1 can act as a conventional, albeit weak, 
transcriptional activator, whereas studies in B lympho-
cytes have emphasized the ability of PU.1 to recruit another 
protein to an adjacent site in an enhancer element (32, 33). To 
examine the enhancer activity of PU.1 in macrophages, we 
assessed the activity in RAW264 cells of CAT expression plas-
mids in which various numbers of PU box motifs are placed 
upstream of a minimal promoter. The plasmids are those used 
in the original descriptions of PU.1 (28). Preliminary experi-
ments revealed that the presence of up to eight PU box motifs 
had little effect on basal transcription activity in RAW264 cells. 
Against this background, we reexamined the effect of a PU.1 
expression plasmid on the activity of the PU box multimers in 
HeLa cells, which had been used previously. Table I confirms 
the finding of Klemza et al. (28) that in HeLa cells, by contrast 
to RAW264, increasing numbers of PU box elements greatly 
increased the basal activity of the promoter, which could be 
amplified further by co-transfection with either PU.1, or an-
other Ets family transcription factor, Ets-2. In RAW264 cells, 
the PU box multimer was less than twice as active as the single 
site control, and it could be activated by co-transfection of an 
Ets-2 expression plasmid but was partly repressed by PU.1.

**Two PU.1 Binding Sites Are Sufficient to Generate a Tissue-
specific Promoter**—The results in the previous section suggest 
that PU.1 alone functions poorly to enhance the activity of a 
TATA-containing promoter in RAW264 cells. In the c-fms pro-
moters (Fig. 1) and in most of the promoters identified in the 
Introduction, the PU box motifs actually occur in the vicinity of 
the transcription start sites. To address directly the alternative 
hyphenation that a binding site for PU.1 alone contributes to 
transcription initiation in a functional macrophage-specific 
promoter, we produced a series of artificial promoters in which 
the high affinity PU.1 binding site from the SV40 virus was 
inserted into the basal promoterless vector pGL2B. This plasmid 
contains a strong polyclonal signal and multiple in 
frame stop codons upstream of the multiple cloning site, so that 
read through transcripts from the plasmid backbone are almost 
undetectable. The plasmids were designated pB, pB>>>, 
pB<>>, and so on, where the arrows signify the orientation and 
number of the PU boxes (the forward arrow signifying the 
purine-rich sequence on the upper strand). Insertion of a single 
PU box site in either orientation had no detectable effect on 
basal promoter activity of pGL2 (data not shown). Conversely, 
pB>> (Fig. 2) had considerable reporter activity in RAW264 
cells; the level of activity of pB>> was similar to the 430-hp 
yhuman fms promoter, approximately 20% of that of the murine 
cytomorphon fms promoter (0.3fms), and 80% of that of the TATA-
containing SV40 minimal promoter in the Promega plasmid, 
pGL2P (data not shown). In NIH3T3 fibroblasts, which lack 
active PU.1 or any other nuclear protein capable of binding 
the PU box sequence under conditions of an electrophoretic 
mobility shift assay (9, 23), the construct had the same barely 
detectable activity as the basal promoterless control vector 
pGL2B. Co-transfection of a PU.1 expression plasmid trans-

### Table I

| Plasmid          | Conversion/μg of cell protein |
|------------------|------------------------------|
|                  | HeLa cells | RAW264 cells |
| PU × 1           | 5.9        | 4.5         |
| PU × 4           | 16.1       | 6.4         |
| PU × 4 plus pECE | 13.4       |             |
| PU × 4 plus pECE | 57.9       |             |
| PU × 8           | 40.6       | 8.8         |
| PU × 8 plus pECE | 35.2       | 6.9         |
| PU × 8 plus pECE | 88.3       | 4.6         |
| PU × 8 plus Ets-pECE | 45.6   | 28.6        |
| pSV2-CAT         | 11.4       | 17.8        |
| pSV2-CAT plus pECE | 8.6   | 14.3        |
| pSV2-CAT plus pECE | 7.3   | 15.1        |
activated the pB≫ construct in NIH3T3 cells but repressed basal activity in RAW264 cells (Fig. 2). Other orientations and combinations of PU sites (pB≫, pB≪, pB≪, pB≪, and pB≪) were examined in transfections of RAW264 and 3T3 cells, but the level of activity and the pattern of trans-activation by PU.1 was always similar to that of pB≫ (data not shown).

PU.1 is not the only Ets factor expressed in macrophages, so we considered the possibility that the requirement for two sites reflects a requirement for cooperation with another Ets factor, as in the interaction between PU.1 and Ets-1 in the immunoglobulin heavy chain enhancer in B lymphocytes (34). Ets-2 is able to recognize and activate via the PU box (Table I) and other PU.1 recognition motifs and has previously been shown to activate the c-fms promoter (9, 23). Co-transfection with Ets-2 expression plasmid transactivated pB≫ in both the RAW264 and 3T3 cells. Co-expression of PU.1 with Ets-2 specifically antagonized this effect in the macrophage line but had an additive effect in the fibroblast line. Control experiments with the parent promoterless vector pGL2B and with the positive control pGL2C (SV40 promoter and enhancer) in RAW264 cells showed that neither was significantly affected by co-transfection of either PU.1 or c-ets-2 expression plasmids (data not shown). Since the pECE expression plasmid used for PU.1 and Ets-2 also contains the SV40 promoter and enhancer, the absence of an effect of PU.1 on pGL2C and on pSV2-CAT (Table I) also argues that the repression of Ets-2 response in RAW264 cells is not due to indirect effects on Ets-2 expression.

Comparative Responses of the Murine and Human c-fms Promoters to Co-transfection of PU.1 and c-ets-2—To ascertain whether PB≫ is a valid model for assessing the function of PU.1, we examined the effects on the human and murine c-fms promoters of coexpressing PU.1 and Ets-2 together in both macrophage and nonmacrophage cell lines. The amounts of co-transfected plasmid (1 μg/10 μg of reporter plasmid) in these experiments were previously determined to produce maximal activation or repression (Refs. 9 and 23; data not shown). In RAW264 cells (Fig. 3A), co-transfection of PU.1 without Ets-2 activated the human but not the murine promoter, while co-transfection of Ets-2 without PU.1 caused a much greater activation of both promoters. As observed with the PB≫ model promoter, co-transfection of PU.1 almost blocked the activation of the murine c-fms promoter by Ets-2 and partly prevented the response to Ets-2 on the human promoter. In NIH3T3 fibroblasts, which lack nuclear PU.1, transfection of the PU.1 or Ets-2 expression plasmids each activated both mouse and human promoters, and the two transcription factors together resulted in a further increase in activity over that obtainable with either factor alone (Fig. 3B). In summary, the behavior of both fms promoters closely resembled the artificial promoter containing only two Ets/PU.1 sites, and maximal activity required that both PU.1 and Ets-2 be expressed.

In previous studies, we showed that the ability of the human promoter to respond to PU.1 and Ets-2 co-transfection was dependent upon the Ets sites in the −150H region that are not well conserved in the mouse promoter (23). Fig. 3C examines the effect of co-transfection with the PU.1 and Ets-2 expression plasmids on the human promoter in which the Ets sites in the −150H region have been mutated. In RAW264 cells, the mutation reduced basal activity, reversed the activating response to PU.1 to a small repression, and also greatly diminished the response to Ets-2 (compare with Fig. 3B). Co-transfection of the PU.1 expression plasmid did not prevent induction of this mutated human promoter by Ets-2. In 3T3 cells, the cooperative response to PU.1 and Ets-2 was retained in the mutated promoter (Fig. 3C). Apart from their direct relevance to understanding the subtle differences between the mouse and human promoters, these studies provide important controls. They confirm that PU.1 can be functionally overexpressed in RAW264 cells and can activate a target promoter through defined cis-
acting elements. They indicate also that the ability of the PU.1 expression plasmid to prevent activation of the murine and human c-fms promoter by an Ets-2 expression plasmid depends upon the presence of specific elements to which both transcription factors can bind.

Elf-1 Can Substitute for c-ets-2 in Transactivating the c-fms Promoter—In previous studies, both Ets-1 and Ets-2 were shown to activate the human c-fms promoter (23). To determine whether these are the only Ets factors able to interact with PU.1 to activate the c-fms promoter, we examined the effect of co-transfection with Elf-1, another Ets factor that is widely expressed in macrophages and other hemopoietic cells (35) and was shown by others (19) to interact with PU box motifs. As with Ets-2, co-transfection with a murine Elf-1 expression plasmid trans-activated the murine c-fms promoter in RAW264 cells (Fig. 4), and the effect was opposed by simultaneous overexpression of PU.1.

DNase I Footprinting of the Murine c-fms Promoter—To explain the response of the mouse c-fms promoter to PU.1 and Ets-2, we identified binding sites for the two factors by DNase I footprinting. For the latter, we again employed the DNA binding domain expressed as a glutathione S-transferase fusion protein because the full-length protein does not bind under conditions of electrophoretic mobility shift assay or DNase footprinting (25). Fig. 5 shows that a clear footprint was observed in the presence of recombinant PU.1, corresponding to the −130M site predicted from the DNA sequence in Fig. 1. A second PU.1 site was observed between −160M and −170M, corresponding to the DNA sequence 5′-GAAAGGGAAC-3′ (on the reverse strand; see Fig. 1). Weak hypersensitivity on the upper strand was observed around the Ets core at −103.

GST-Ets-2 caused a modest reduction in the intensity of two bands in the core of the −130M site on the upper strand. On the lower strand, GST-Ets-2 caused an extended change in band intensities in the region from −110M to −133M. The hypersensitivity to DNase I caused by both PU.1 and Ets-2 may reflect the observation that binding of Ets factors with DNA

![Fig. 3. Transient transfection analysis of the effect of coexpressed PU.1 and Ets-2 on the murine and human c-fms proximal promoters in RAW264 macrophage-like cells (A) and NIH3T3 fibroblasts (B). Transfections and luciferase assays were performed as described under “Materials and Methods.” Results are the average of duplicates; the experiment is representative of three. In panel A, although it is not evident because of the vertical scale, co-transfection with PU.1 caused a 5.1-fold increase in basal activity of the human promoter as described elsewhere (23). C, experiments in panels A and B were repeated using the −150H PU/Ets mutant of the human −430 fms promoter described previously (23). Results are the average plus or minus S.D. of three experiments, each performed in duplicate. The small repression by PU.1 of the Ets-2 response in RAW264 cells was observed in all three experiments but is not statistically significant.](http://www.jbc.org/content/275/5/6654)

![Fig. 4. Transient transfection analysis of the effect of coexpressed PU.1 and Elf-1 on the murine c-fms proximal promoter (pGL0.3fms) in RAW264 macrophage-like cells. Transfections were carried out as described under “Materials and Methods.” The results are the average of duplicates; the experiment is representative of three.](http://www.jbc.org/content/275/5/6654)
Ets Sites in Macrophage-restricted Basal Transcription

**DISCUSSION**

This study shows that PU.1 recognition sites are weak enhancer elements in RAW264 macrophages (Table 1), but alone they are sufficient to activate a minimal promoter in the absence of a TATA box or consensus initiator element (Fig. 2). One PU.1 binding site was insufficient to generate detectable promoter activity, whereas a basal vector that contains only two PU box motifs (pB++) resembled the murine and human c-fms promoters in several important respects: (i) like the c-fms promoters, pB++ displayed promoter activity that was dependent upon endogenous or co-transfected PU.1 expression; (ii) pB++ could be activated by coexpressed Ets-2 in both RAW264 macrophage-like cells and NIH3T3 fibroblasts in the same way as the c-fms promoters; (iii) trans-activation of both c-fms promoters and pB++ in RAW264 cells with Ets-2 was antagonized by simultaneous coexpression of PU.1, whereas these factors produced an additive effect in NIH3T3 cells.

Based upon these observations, we hypothesize that the reason that the c-fms promoters (Fig. 1) and all of the myeloid-specific promoters discussed in the Introduction have more than one purine-rich proximal promoter element is that maximal activity requires that one Ets site be occupied by PU.1 and another by a second Ets factor exemplified by Ets-2. According to this model, PU.1 can act as a repressor of the response to another co-transfected Ets factor because it prevents binding to the second site. This occurs when PU.1 is co-transfected with the reporter plasmid in RAW264 macrophages, where there is already a very high basal level of nuclear PU.1 (9) but not to the same extent in the transfected 3T3 fibroblasts when the expression of both of the Ets factors is directed by the SV40 globulin heavy chain enhancer, presumably by competing with specific domains of other Ets proteins, and to act as a repressor of Ets-1 or Ets-2 actions has several precedents. Erman and Sen (34) showed a clear instance of context-dependent cooperation between PU.1 and Ets-1 on the immunoglobulin heavy chain intronic enhancer, where specific domains of each protein are required for transactivation to occur. Overexpression of PU.1 in B cells causes repression of the immunoglobulin heavy chain enhancer, presumably by competing with Ets-1 for occupancy of the second site (9). Others showed that

![Fig. 5. DNase I footprint analysis of the proximal murine c-fms promoter with recombinant PU.1 (black) or recombinant GST-Ets-2 (shaded). Profiles for upper (MluI-labeled) or lower (HindIII-labeled) strands are shown. Lanes 1 and 3 are identical and were duplicated to facilitate comparison. Protected regions are marked on the profile (and on the sequence below) with bars, while hypersensitive sites are marked with open circles. Numbering is backwards from the translation start codon.](Image 69x374 to 278x729)

![Fig. 6. Transient transfection analysis of the function of the −103M putative Ets site in RAW264 cells. The mutation (GGAA → CGAA) of the core Ets binding motif was carried out as described under “Materials and Methods.” Wild-type and mutant (CC) reporter plasmids and the promoterless control pGLB were transfected into RAW264 cells, and luciferase was assayed as described. Results are the average of duplicates and are representative.](Image 323x558 to 539x729)
PU.1 represses activation through an Ets motif of the class II major histocompatibility complex I-A promoter in B cells (37). By contrast to PU.1, Ets-2, is a powerful trans-activator (Table I). It is capable of activating transcription in RAW264 cells through a single response element (25). Hence, the function that Ets-2 contributes to both PB>> and the c-fms promoters may be a strong activation domain that is not present in PU.1.

The model promoter gives insight into the possible functions of PU.1 but does not indicate how the direction of transcription and the start sites are determined. The −103M site was considered as an additional candidate Ets site in the c-fms promoters based upon conservation of the Ets core between mouse and human, its position flanked by the major transcription start sites (Fig. 1), and the fortuitous presence of a similar sequence in the same position relative to the PU boxes in the model promoter, pB>>. If the PU box motifs function as the equivalent of a TATA box, as hypothesized in the introduction and supported by the transfection data, then the −103M site is appropriately placed to be the equivalent of an initiator. Mutation of the core Ets site (GGAA to CCAA) abolished promoter activity, demonstrating that this is an additional site that is absolutely required for the activity of the murine fms promoter (Fig. 6). The −103M motif is found in the basal promoters of several other myeloid genes. Both the human GM-CSF receptor (18) and the human CD18 (12–14) promoters have exactly the same spacing of a distal PU.1 site and a GGAA motif adjacent to the major transcription start sites (12–14). In the c-fes promoter the major sites of transcription initiation also occur around the same sequence as in murine c-fms, CAGGAAC (19).

In the macrophage-specific promoter of the PU.1 gene itself, in both mouse and human, the identical sequence again lies within the transcription start region, and the same CC mutation we made in the fms promoter greatly reduced activity of the PU.1 promoter in myeloid cells (22). On the basis of these many examples, a GGAA core sequence can be viewed as a myeloid-specific initiator sequence. In none of the examples cited does the Ets-like initiator sequence bind PU.1 with high affinity. The CAGGAAC site in the PU.1 promoter can bind recombiant PU.1 weakly in an electrophoretic mobility shift assay (22), an observation we have confirmed with the c-fms sequence (data not shown). Recombinant PU.1 caused a weak hypersensitivity on the upper strand of the c-fms promoter in the DNase I footprinting shown in Fig. 5. It may be that the site must be weak so as to reduce competition by other Ets factors that would not optimally fulfill the function provided by PU.1 or to provide a sensitive relationship between the level of PU.1 expressed and c-fms transcription. A similar argument has been made to explain the weak binding of Elf-1 to proximal promoter elements in T cell-specific promoters (38). DNase I footprinting using RAW264 macrophage nuclear extract does not detect proteins bound to this site; instead, the whole region becomes hypersensitive to digestion.2 This observation cannot be interpreted, since some full-length Ets factors bind weakly if at all to DNA in vitro or form multimeric complexes with other factors, such as members of the API family, bound to adjacent sites (1, 20, 25, 39, 40). Hence, it remains possible that PU.1 itself binds alone, or cooperatively with another factor, as in the case of B-cell-specific elements (32, 33) or that another Ets-like factor that cannot be detected by the methods employed in this study contributes to the function of the −103M element.

PU.1 cooperates with Ets-2 to activate both the mouse and human c-fms promoters in 3T3 cells, but the promoters from the two species differ in basal activity and response to PU.1 co-transfection in RAW264 cells. Both species have at least three Ets core sequences: the −103M/−105H site discussed above and PU.1 binding sites at −130M/−150H and −170M/−170H. The −150H site differs from the −130M site in that it actually contains three possible GGAA or GGAT Ets cores (Fig. 1). These sites are required for the human-specific activation of the fms promoter by co-transfection of PU.1 and the maximal response of the human promoter to Ets-2 in RAW264 cells (Ref. 23; Fig. 3C). The −150H site(s) have a lower affinity for PU.1 than the distal −170H site (23), and were not detected in DNase I footprinting (10). Overexpression of PU.1 might permit a higher level of occupancy of the low affinity −150H sites, leading to the observed transcriptional activation (Fig. 3C). By contrast to the human promoter, the mouse has two strong PU.1 sites. The region surrounding the high affinity PU.1 binding site at −170M, identified in this study by DNase I footprinting (Fig. 5), can be clearly aligned between mouse and human (Fig. 1), but the GGAA Ets core is not conserved. Whereas the mouse sequence is 5′-GAAAGGAACT-3′ (reverse strand), the human sequence is 5′-GCAAGGCACCC-3′. Functionally, therefore, the −170M site might be more accurately aligned with −170H, although the PU/Ets site is in the reverse orientation. It remains to be seen whether the subtle differences in PU.1 dependence and relative activity of the human and mouse fms promoters are related to different roles of c-fms and its ligand in macrophage differentiation between the two species.

The use of Ets-2 as a model for a second Ets factor in this study was prompted by the previous evidence of the response of the mouse and human fms promoter to this factor (9, 23) and its known ability to interact with PU box elements (Table I). It is unlikely to be the only factor that can interact with PU.1 to activate myeloid promoters. However, the relevance of the model is supported by evidence that Ets-2 can promote macrophage differentiation when overexpressed in premyleloid cells (41), and a dominant negative Ets-2 expressed from the human c-fms promoter caused significant aberrations in macrophage differentiation in vivo (42). Ets-2 protein is expressed in primary macrophages; the level of expression is regulated by CSF-1 and phorbol esters (25, 40, 42), but Ets-2 itself is not essential for the differentiation of normal primary macrophages from embryonic stem cells (4). Given the size of the Ets family, a degree of redundancy is not surprising. We showed that Elf-1 mimicked the effect of Ets-2 (Fig. 4). Elf-1 is expressed constitutively in primary murine macrophages (35).

Heydemann et al. (19) described a proximal promoter element in the myeloid-specific c-fes promoter that binds PU.1 or Elf-1 with equal affinity, and both proteins were shown to be expressed at similar levels in the nuclei of human myeloid cells. Like Ets-2, Elf-1 is also not absolutely required for myeloid differentiation (19), but another Elf-1-related protein, termed MEF, recently described in murine myeloid cells (43), could also contribute. In examining the relevance of the effects of these co-transfected Ets factors, it is important to recognize that, although RAW264 is the best available transfectable murine macrophage cell line model, the level of c-fms expressed is low in comparison with bone marrow-derived macrophages (25) and lower still than in postproliferative peritoneal macrophages.2 RAW264 cells and primary macrophages have similar levels of nuclear PU.1 (9), but the cell line lacks Ets-2 mRNA and protein (25) or nuclear protein that binds strongly to a perfect Elf-1 consensus site.3 Increasing levels of Ets-2 or other factors during later stages of macrophage differentiation could contribute to increased expression of c-fms.

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2 X. Yue and D. A. Hume, unpublished observations.
3 D. A. Hume, unpublished observations.
4 D. A. Hume and M. C. Ostrowski, unpublished observations.
5 I. L. Ross and D. A. Hume, unpublished observations.
It is of some interest that Ets-2 alone can activate both the pBΔ model promoter and the c-fms promoter in 3T3 cells (Figs. 2 and 3), which lack PU.1 (9, 23). The c-fms promoter is active in a wide range of mouse and human tumor cell lines, and ectopic expression of c-fms, which commonly creates a CSF-1/c-fms autocrine loop, is a feature of malignant mouse and human tumor cells that is correlated with invasive potential and anchorage-independent growth (see Ref. 44 and references therein). The c-fms proximal promoter is actually growth factor-dependent and can be activated by both CSF-1/c-fms (44) and GM-CSF/GM-CSF receptor (45) signaling on a normal fibroblast background. In turn, CSF-1/c-fms signaling in 3T3 cells requires Ets-2 and can be blocked by expression of dominant negative forms of the protein (25, 40). Expression of c-fms mRNA, which is restricted to macrophage-like cells in the mouse embryo (46) remains detectable in the fetal liver of PU.1 null mice (47). These findings together suggest that the fms promoter is not absolutely PU.1-dependent, and other Ets factors such as Ets-2 are able to substitute for PU.1 activity, albeit less effectively.

Clearly, PU/Ets sites alone do not explain the full complexity of expression of c-fms or any other macrophage-specific gene; the PBΔ model applies only to the basal promoter and initiation. One other factor that regulates the c-fms promoter is c-myb, which represses promoter activity and is down-regulated as c-fms is induced during myeloid differentiation (23). CBFα/AML1, which is also tissue-restricted, cooperates with CBFα and CAAT enhancer-binding protein α to activate the human c-fms promoter in myeloid cell types (2). Interestingly, the sites involved in this response are not conserved in the mouse (Fig. 1). The two sequence differences that create the second high affinity PU.1 binding site in the mouse at −170M, actually change two key bases in the human CBFα/B site (human GTGGTTG; mouse GTAGTTC), but despite these changes the mouse promoter is responsive to trans-activation by another member of the CAAT enhancer-binding protein family that has also been implicated in myeloid differentiation, CAAT enhancer-binding protein β.

The kinds of interactions between PU.1 and Ets-2/EIf-1 observed on the PBΔ and c-fms promoters are specific to those promoters. Although PU.1 is already expressed at high levels in RAW264 cells, the ability of this factor to activate the human c-fms promoter, under the same conditions where it represses the mouse promoter, indicates that it is not saturating for weaker PU.1 box sites. In other studies, we have shown that co-transfection with the PU.1 expression plasmid can activate the human immunodeficiency virus-1 long terminal repeat in RAW264 cells. This activation is dependent upon the tandem NF-B sites, which bind weakly to PU.1 in vitro (48, 49). The same sites also function as Ets-2-responsive elements, but specific mutations that prevent PU.1 (and NF-B) binding and activation do not block Ets-2 action (49). Furthermore, PU.1 does not prevent activation by Ets-2, further indicating that the ability of PU.1 to block Ets-2 action on the mouse and human c-fms promoters is not due to nonspecific effects on Ets-2 expression or function under the conditions of transfection. Another promoter that responds to both factors is the tartrate-resistant acid phosphatase promoter, a TATA box-containing promoter functional in osteoclasts. In another study, we have shown that PU.1 co-transfection activates this promoter 5–10-fold in RAW264 cells via a weak PU box site in the proximal promoter. In contrast to these promoters, the CSF-1-inducible promoter of the urokinase plasminogen activator gene is responsive only to Ets-2, which acts via a conserved Ets/AP1 element in a distal enhancer that does not bind PU.1 (25). It is important that such elements do not bind PU.1, since Ets-2 is a key component of the ras/raf/mitogen-activated protein kinase signaling pathway (40) and competition by PU.1 could prevent the operation of this pathway in macrophages. These observations highlight the fact that the interactions between PU.1 and other Ets family factors depend upon promoter context, the relative affinity of the binding sites for different Ets factors, and the level of expression. An additional complexity may arise based upon recent observations that PU.1 can be inducibly phosphorylated in macrophages (49) and that a significant proportion of macrophage PU.1 is found in the cytoplasm.

In this study, we have provided evidence that the multiple purine-rich motifs found in many macrophage-specific proximal promoters contribute different functions to transcription initiation. We have shown that PU.1 is not the only factor that binds them and that PU.1 is not sufficient, and may not be necessary, for maximal promoter activity. In essence, the archetypal basic myeloid promoter probably has three factors bound to purine-rich motifs: PU.1, a second Ets factor with a powerful activation domain, and a third factor/complex that interacts with the GGAA initiator sequence. The hypothesis derived from our studies of the PBΔ and fms promoters should have general relevance to understanding macrophage differentiation and the functions contributed by PU.1 and other Ets factors to the activity of myeloid-specific TATA-less promoters.

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