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TFEC Is a Macrophage-Restricted Member of the Microphthalmia-TFE Subfamily of Basic Helix-Loop-Helix Leucine Zipper Transcription Factors

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The murine homologue of the TFEC was cloned as part of an analysis of the expression of the microphthalmia-TFE (MiT) subfamily of transcription factors in macrophages. TFEC, which most likely acts as a transcriptional repressor in heterodimers with other MiT family members, was identified in cells of the mononuclear phagocyte lineage, coexpressed with all other known MiT subfamily members (Mitf, TFE3, TFEB). Northern blot analysis of several different cell lineages indicated that the expression of murine TFEC (mTFEC) was restricted to macrophages. A 600-bp fragment of the TATA-less putative proximal promoter of TFEC shares features with many known macrophage-specific promoters and preferentially directs luciferase expression in the RAW264.7 macrophage cell line in transient transfection assays. Five of six putative Ets motifs identified in the TFEC promoter bind the macrophage-restricted transcription factor PU.1 under in vitro conditions and in transfected 3T3 fibroblasts; the minimal luciferase activity of the TFEC promoter could be induced by coexpression of PU.1 or the related transcription factor Ets-2. The functional importance of the tissue-restricted expression of TFEC and a possible role in macrophage-specific gene regulation require further investigation, but are likely to be linked to the role of the other MiT family members in this lineage. The Journal of Immunology, 1999, 162: 1559–1565.

Monocytes, macrophages, and their precursors represent different stages of differentiation in a functionally diverse hemopoietic cell lineage (1). The mature endpoints of this lineage not only include the heterogenous group of tissue macrophages, but also two other specialized cell types: the multinucleated bone-resorbing osteoclast and the Ag-presenting dendritic cell (2, 3). Differentiation of common progenitor cells to particular fates is thought to be under the stringent control of the surrounding microenvironment and local tissue-specific factors, acting upon specific transcription factors that in turn direct lineage-specific gene expression. In vitro culture systems that allow the generation of osteoclast- or dendritic-like cells from monocytes reflect the complex structure of this process (3–5).

In recent years, the targeted disruption of several genes (e.g., PU.1, c-fos, c-src) and the molecular characterization of natural mutations (op/op, mi/mi) have led to new insights into developmental processes and functions of macrophages and osteoclasts (6–10). In op/op and PU.1−/− mice, both cell types are severely affected, probably at the stage of a common precursor. The osteopetrotic op/op mouse carries an inactivating mutation of CSF-1 (macrophage CSF); an important survival and differentiation signal for macrophages and osteoclasts normally provided from the surrounding tissue (9). The receptor for CSF-1, c-Fms, is itself macrophage/osteoclast specific (11), and in the absence of CSF-1, macrophage and osteoclast numbers are severely reduced (12). PU.1 is an Ets family transcription factor (13) that binds key elements of macrophage-specific promoters, including that of c-fms (14), and the targeted disruption of the transcription factor PU.1 demonstrated its importance for the development of both cell types (6, 15, 16).

Three mutations of the transcription factor Mitf selectively affect osteoclast development or function and result in osteopetrosis (17, 18). Mitf, which is encoded by the microphthalmia gene (10), is a member of the large family of basic helix-loop-helix leucine zipper (bHLH-ZIP) transcription factors and, like other bHLH-ZIP proteins, forms homo- or heterodimers that bind a cognate DNA sequence termed the E box (CANNTG) (19). Sequence analysis and biochemical studies demonstrated a close relationship between Mitf and the three transcription factors, TFE3 (20), TFEB (21), and TFEC (22, 23), which are less well understood in terms of expression pattern and function. Together, these four transcription factors form the Mit subfamily of bHLH-ZIP transcription factors, and they have been shown to form heterodimers with each other, but not with known bHLH-ZIP factors of other subfamilies (19). Mitf itself is known to be expressed in melanocytes and to bind various E boxes with an extended site, termed M boxes, in promoters of several melanocyte-specific genes, e.g., tyrosinase, tyrosinase-related protein-1, and tyrosinase-related protein-2 (24–26). Numerous mutant alleles of the murine microphthalmia gene

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3 Abbreviations used in this paper: bHLH-ZIP, basic helix-loop-helix leucine zipper; BMM, bone marrow-derived macrophage; EMSA, electrophoretic mobility shift assay; hTFE, human TFE; mTFE, marine TFE; Mit, microphthalmia-TFE; nt, nucleotide; OLC, osteoclast-like cell; RACE, rapid amplification of complementary deoxyribonucleic acid ends; TGE-PM, thioglycolate-elicited peritoneal macrophage.
have been characterized that all affect pigment-producing melanocytes (17). By contrast, only a subset of Mitf mutations affects cells of hemopoietic origin (mast cells, osteoclasts, NK cells). These mutations are subtle variants that produce dominant-negative proteins that retain their ability to homo- and heterodimerize, but cannot bind to DNA (27). An obvious corollary is that osteoclasts express dimerization partners for Mitf.

Macrophages with mutations in Mitf have not been studied very thoroughly, and dramatic functional or developmental defects in macrophages have not been reported, with the exception of a reduction of superoxide dismutase activity (28). Hence, the most obvious explanation for the absence of an overt phenotype in the macrophage lineage of mi/mi is that the level or pattern of expression of Mitf dimerization partners renders them resistant to the dominant-negative gene. Of course, the other alternative is that Mitf transcription factors have no function in the macrophage lineage.

To address this issue and to investigate a possible role of Mitf subfamily transcription factors in macrophages, we aimed to determine which Mitf members are expressed during differentiation of the monocyte-macrophage lineage and how their expression is controlled.

In this study, we show that all four known members of the Mitf family are expressed in macrophages, including Mitf, TFE3, TFEB, and the murine homologue of TFEC, which we cloned during this study. Interestingly, the expression of murine TFEC appeared restricted to cells of the monocyte-macrophage lineage. We show that the tissue-restricted expression of mTFEC is controlled by a typical TATA-less, macrophage-type promoter that contains several Ets motifs that bind the myeloid and B cell-restricted transcription factor PU.1. These findings establish mTFEC as a macrophage-specific transcription factor that might itself, individually or in concert with Mitf, TFE3, and TFEB, play a role in macrophage-specific gene regulation.

Materials and Methods

Chemicals

All chemical reagents used were purchased from Ajax Chemicals (Auburn, Australia), unless otherwise noted. Oligonucleotides were synthesized by Pacific Oligo (Lismore, Australia).

Cell culture

All cell lines used in this study were obtained from the American Type Culture Collection (Manassas, VA). The cell lines J774, WR19 M1, WR19L, EL-4, MPC-11, MOPC31C, B16, and NIH3T3 and L929 were maintained in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS (Life Technologies). RAW264.7 and M1 cell lines were cultivated in RPMI 1640 medium (BioWhittaker, Walkersville, MD) plus 10% FCS. Murine bone marrow–derived macrophages (BMMs) and thioglycolate-elicited peritoneal macrophages (TGE-PMs) were obtained and cultured as described previously (29). Osteoclast-like cells (OLCs) were generated in a coculture system of primary mouse calvarial osteoblasts with spleen cells in the presence of 1.25-dihydroxyvitamin D₃, as described by TAKAHASHI et al. (5).

RNA preparation and Northern blot analysis

Total RNA was isolated from different cell lines by the guanidine thiocyanate/acid phenol method (30). Electrophoresis, Northern blotting, and cDNA hybridization were conducted as described previously (31). The probes used were: a 1200-bp cDNA of the mTFEC coding region, an 816-bp PU.1 cDNA, and an oligonucleotide complementary to mouse 18S rRNA.

PCR amplifications

Total RNA from BMMs and in vitro differentiated osteoclasts was reverse transcribed and, together with degenerate primers (bHLHZIP–S–AS; Fig 2), used as a template to amplify the conserved bHLH-ZIP region of MIT subfamily members. Conditions were 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min, repeated for 35 cycles. Amplified cDNA fragments were subcloned, and several independent colonies were used for restriction and sequencing analysis.

The amplification of specific fragments for MIT transcripts was done using the Expand High Fidelity PCR system (Boehringer Mannheim, Indianapolis, IN). Products were sequenced to confirm their identity. Primer positions and fragment sizes are indicated in Fig. 1.

For the cloning of 5'- and 3'-cDNA fragments for mTFEC, a modified RACE-PCR approach was used. Full-length cDNA from total BM RNA was generated with the SMART-PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA), according to the manufacturer’s instructions. The cDNA containing the SMART oligonucleotide, which is added to the 5’ end of full-length mRNA transcripts during reverse transcription, was used to amplify 5'- and 3'-cDNA fragments for mTFEC. Nested PCR with gene-specific primers (5’R1, 5’R2') and a 5’PCR primer complementary to the SMART oligonucleotide yielded an 850-bp fragment representing the full-length 5’ end of TFEC, which was subcloned. Two independent clones were sequenced and contained identical 5’ ends. A similar approach using gene-specific primers 3’R1 and 3’R2 and a 3’-oligo(dT) primer allowed the isolation of a 1.4-kb TFEC 3’-end fragment.

To amplify genomic fragments of the proximal promoter region of mTFEC, we used nested gene-specific primers (IN, OUT) and the Promoter Finder DNA Walking Kit (Clontech), following manufacturer’s instructions. Positions and sequence of oligonucleotides used for cloning purposes are indicated in Fig. 2.

DNA sequence analysis

The cDNA sequencing was done by Dye Deoxy Terminator Cycle Sequencing (Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions, and sequences were analyzed on the Applied Biosystems DNA Sequencing System (model 373A) by the Australian Genome Research Facility.

Plasmid construction and purification

PCR fragments were inserted into the plasmid vector pCR2.1 (TA Cloning Kit; Invitrogen, San Diego, CA) for sequencing and subcloning purposes. To generate the pGL2-TFEC reporter plasmid, a 615-bp fragment of the TFEC proximal promoter was subcloned into pGL2-B (Promega, Madison, WI) cut with XhoI and HindIII. The mouse PU.1 expression plasmid pECE-PU.1 and mouse Ets-2 expression plasmid pECE-Ets-2 were a gift from Dr. Richard Maki, (Barnburn Institute, La Jolla, CA). For transient transfections, plasmids were isolated by the alkaline-SDS method and purified by two subsequent CsCl density-gradient centrifugations (32).

Transient DNA transfections

NIH3T3 fibroblasts and the RAW264.7 cell line were transfectected using Superfect reagent (Qiagen, Chatsworth, CA), according to the manufacturer’s instructions. Briefly, 5 × 10⁶ NIH3T3 cells or 8 × 10⁶ RAW264.7 cells were seeded into six-well plates the day before transfection. A total of 10⁴ NIH3T3 cells or 8 × 10⁶ RAW264.7 cells were transfected with 0.6 µg reporter plasmid and 10 µl Superfect reagent was used to transfect the cells. In cotransfections, 0.2 µg of each expression plasmid and/or control plasmid was added to a total of 1 µg DNA. After 2 h, the cells were washed and supplemented with the appropriate medium. The transfected cells were cultured for 22 h and harvested, and cell lysates were assayed for luciferase activity (Luciferase Reporter Gene Assay; Boehringer Mannheim) on a 1450 MicroBeta TRILUX (Wallac, Gaithersburg, MD). Activity was normalized to protein concentration measured using a modified Bradford assay (Bio-Rad) or in the case of cotransfections on cell number.

Nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared with a variation of the method of Osborn et al. (33). pPU.1 was prepared and purified as described elsewhere (34). Oligonucleotides were end labeled with [γ-³²P]ATP using T4 polynucleotide kinase. For the oligonucleotides etbox1–6 and PU box, the binding reaction contained 2.5 µg of nuclear extract protein, 1 µg of poly d(IC), 20 mM HEPES, pH 7.9, 40 mM KCl, 1 mM DTT, 1 mM EDTA, pH 8, 5% glycerol, and 40 nmol of probe DNA in a final volume of 10 µl. Samples were loaded onto 10% polyacrylamide gels after standing at room temperature for 30 min. Buffers and running conditions used have been described (34). Gels were fixed in 10% acetic acid, dried, and autoradiographed.
Results

MiT subfamily gene expression in macrophages and osteoclasts

To assess the expression of MiT subfamily members, we initially performed reverse-transcriptase PCR with degenerate primers covering the conserved basic helix-loop-helix region. A 269-bp fragment was generated in murine BMMs and OLCs, cloned, and analyzed by sequencing and restriction mapping (data not shown). Along with several fragments corresponding to the known murine TFE3 and Mitf homologues, we identified fragments for a murine homologue of rat and human TFEC (22, 23). Use of a corresponding control template DNA for TFEB showed that it was less efficiently amplified with the degenerate primers; therefore, we also used specific primers to analyze for MiT subfamily gene expression in macrophage and nonmacrophage cell types. As shown in Fig. 1, expression of all four members could be detected in BMMs, TGE-PMs, and OLCs. The fragment for TFEC could only be amplified in macrophage cell types and to a much lesser extent in OLCs. The primers used to amplify TFE3 were designed to allow the separation of the two isoforms, mTFE3-L, and mTFE3-S. The latter lacks the exon encoding the acidic domain that corresponds to a transcriptional activation domain of mTFE3-L and acts as a transcriptional repressor (35). In all cases, the larger fragment for the transactivator TFE3-L was the predominant amplified product.

Sequence analysis of the murine homologue of TFEC

The restricted expression pattern of TFEC had not previously been described. To allow further analysis of the murine homologue of TFEC, a full-length cDNA was cloned by a modified RACE-PCR approach that allows the 5'-mRNA end-dependent, selective amplification of full-length transcripts. Identical start sites for two independent 5' fragments indicate a major cap site at the first nucleotide of the cDNA. The complete sequence is shown in Fig. 2. The deduced amino acid sequence of the putative mTFEC protein shows 90% identity with rTFEC and 77% identity with hTFEC-L. Similar to its homologue in rat, the sequence of mTFEC lacks the exon encoding an acidic domain that is present in the human homologue of TFEC (22, 23). This exon corresponds to a transcriptional activation domain of subfamily members TFE3 and Mitf (36, 37). PCR amplification of a putative corresponding exon in nTFEC revealed no products for a longer isoform that would include the activation exon (data not shown).

Macrophage-restricted expression pattern of mTFEC

Northern blot analysis of a wide range of cell lines confirmed our initial observation that the expression of mTFEC is restricted to cells of the mononuclear phagocyte lineage. As shown in Fig. 3A, a 1.8-kb RNA transcript was detectable in BMM, TGE-PM, and the macrophage cell lines RAW264.7 and WR19 M.1, but not in any of the other cell types, including T cell (WR19L, EL-4), B cell (MPC-11, MOPC31C), melanocyte (B16), and fibroblast cell lines (NIH3T3, L929). A 3-kb RNA species that had been shown to hybridize with a probe for rat TFEC could not be detected (22). In OLCs, the message was barely visible in Northern blot analysis, but in contrast to nonmyeloid cells, could be detected by PCR (see Fig. 1). The same blot was probed for expression of the Ets family transcription factor PU.1 that was expressed in macrophages, osteoclasts, and B cells (Fig. 3A). To confirm the association of TFEC with macrophage differentiation in primary cells, a time course of total RNA was prepared from bone marrow cells cultured with CSF-1. Within the limits of detection of Northern blot analysis, PU.1 was induced rapidly, whereas TFEC was induced later in the differentiation process corresponding to markers such as c-fms (Fig. 3B).

Isolation and macrophage-specific activity of the proximal TFEC promoter

The expression pattern suggested that TFEC might be a macrophage-specific member of the MiT subfamily of BHLH-ZIP

FIGURE 1. Gene expression of MiT family members. Reverse-transcriptase PCR was performed on RNA from indicated cell types using gene-specific primers for Mitf (nt 464..487, nt 847..873; Accession Number Z23066), TFE3 (nt 71..97, nt 729..755; Accession Number S76673), TFEB (nt 729..754, nt 1113..1134; Accession Number AF079095), TFEC (nt 557..581, nt 876..900; Accession Number AF077742), and β-actin (nt 414..435, nt 781..808; Accession Number X03672). PCR reactions for individual genes were performed in parallel, with increasing cycle numbers to allow for an analysis in the exponential range.

FIGURE 2. cDNA sequence of mTFEC, 5' and 3' fragments of the mTFEC transcript were amplified by a modified RACE-PCR technique, cloned, and sequenced. The complete cDNA is shown and contains an open reading frame encoding a protein of 317 amino acids in length. The basic helix-loop-helix region is marked as a grey box, the leucine residues of the zipper as grey spheres. The position of a consensus polyadenylation site is underlined, and sequences and positions of primers used for cloning purposes are indicated.
transcription factors. In many cases, the basal tissue-restricted expression of genes is controlled by cis-acting elements located within the 5′-proximal promoter, so we cloned and sequenced a corresponding region of the murine TFEC gene. Promoter Finder DNA Walking (Clontech) was used to isolate a 615-bp genomic fragment of the 5′-proximal TFEC promoter that was subsequently sequenced and analyzed for putative regulatory elements. The sequence, as shown in Fig. 4, shares remarkably similar features with the promoters of several genes that are specifically expressed in macrophages (e.g., c-fms (14, 34), PU.1 (38), CD11b (39), CD18 (40), FcyRI (41, 42), FcyRIII (43), c-fes (44), the macrophage scavenger receptor (45), and Nramp-1 (natural resistance-associated macrophage protein) (46)). As in the promoters of the above listed genes, the 5′-proximal region of TFEC is characterized by the absence of TATA boxes, consensus initiator sequences, or GC-rich regions found in housekeeping genes that normally determine transcriptional initiation. It also lacks Sp1 or CCAAT-box sequences, and instead contains six motifs that might be recognized by transcription factors of the Ets family, including PU.1 (47). Other identified motifs include putative binding sites for nuclear factor-kB, C/EBP-β, c-myc, and AP-1, which might also be important for myeloid-specific gene expression in some cases (45, 48–52).

The sequence structure of the proximal promoter suggested that this region might be implicated in the tissue-restricted expression of the TFEC gene. To test this possibility, we inserted the TFEC-promoter fragment into the promoterless vector pGL2-B and assayed its reporter activity in transient transfections. Whereas in NIH3T3 fibroblasts the construct was only slightly more active than the promoterless control vector, it was effectively activated in the macrophage cell line RAW264.7, approximately 60 times over the activity of pGL2-B (Fig. 5).

**PU.1 binding and transactivation of the TFEC promoter**

Growing evidence indicates an important role of PU.1 recognition motifs for the expression of macrophage-restricted genes (14, 29, 38, 39, 43, 45). Six Ets-like sequences are present in the TFEC promoter that could possibly bind PU.1. Fig. 6 shows EMSA using double-stranded oligonucleotides of corresponding Ets sequences in the TFEC promoter. Four of these sequences (etsbox-1, -3, -4, -6) competed with the binding of rPu.1 to the Pu-box oligonucleotide from the SV40 enhancer (Fig. 6A) that represents a known, high affinity binding site for Pu.1 and was used as a reference. Subsequently, efficient direct binding of rPu.1 was demonstrated for etsbox-1, -3, -4, and -6 oligonucleotides (Fig. 6B), whereas etsbox-2 was only weakly bound. No Pu.1 binding of etsbox-5 was detectable. Etsbox-6 represented the highest affinity binding site. Compared with the SV40 enhancer binding site, all four binding sites were less effectively bound by Pu.1. Similar results were obtained using nuclear extracts from RAW264.7 cells (data not shown). The specific binding of each oligonucleotide to Pu.1 in nuclear extracts was further established by competition with cold Pu-box oligonucleotide. The binding activity of this major complex was reduced, and its mobility was slightly increased by addition of preimmune serum, but was selectively abolished by specific anti-Pu.1 antiserum. Fig. 6C shows a representative EMSA using etsbox-6 oligonucleotide and nuclear extracts from RAW264.7 cells. Similar results were obtained for etsbox-1, -3, and -4. No binding of nuclear proteins could be detected with etsbox-2 or -5 oligonucleotides (data not shown).

Our group has shown recently that in NIH3T3 fibroblasts, which lack nuclear Pu.1, the expression of Pu.1 and/or Ets-2 activated

**FIGURE 4.** Sequence of the proximal promoter region of TFEC. A genomic fragment of the 5′-proximal TFEC promoter was cloned via Promoter Finder DNA Walking and sequenced. Putative binding sites for several transcription factors that were identified using the TRANSFAC database (57) are indicated. Dashed lines mark the positions and sequences of etsbox-1–6 oligonucleotides, which were used for EMSA.

**FIGURE 5.** The TFEC promoter is activated specifically in macrophages. The fibroblast cell line NIH3T3 and the macrophage cell line RAW264.7 were transfected with the TFEC-promoter construct, as described in Materials and Methods. Values represent the luciferase activity relative to the empty control vector pGL2-B, and are the mean + SD obtained from three independent experiments.
reporter constructs containing the proximal promoters of human and mouse c-fms or an artificial minimal promoter consisting of two copies of the SV40 PU-box element (29). As shown in Fig. 7, both PU.1 and Ets-2 were also able to activate the TFEC promoter construct, and coexpression of PU.1 with Ets-2 had an additive effect in the fibroblast cell line.

Comparison with other macrophage-type promoters

Fig. 8 highlights major characteristics found in the region of transcriptional initiation of a range of well-characterized myeloid-specific promoters. The aligned sequences generally contain multiple Ets-like recognition motifs. In many cases, these purine-rich sequences have been shown to be strictly necessary for basal promoter activity. The comparison also indicates another common, yet uncharacterized, motif (CCAGTG) that can be found in many myeloid promoters. This region will be subject to further investigations. Initial experiments do not indicate the binding of macrophage-restricted proteins from nuclear extracts to this site. The macrophage-specific promoters of c-fms and TFEC share the strongest similarity, including number and position of Ets-like motifs. A weak Ets-like binding motif (CAGGAA) that is essential for the activity of the c-fms promoter (29) and the promoter of PU.1 (38) itself is also present in the TFEC promoter at a similar position.

Discussion

In this study, we present evidence for a novel macrophage-restricted transcription factor. We cloned the murine homologue of TFEC during an attempt to characterize the MiT subfamily of bHLH-ZIP transcription factors in macrophages, and show in this work that its expression is highly restricted to cells of monocyte/macrophage origin. Characterization of the putative proximal promoter of mTFEC reveals typical macrophage-specific features and supports placement of TFEC within the group of macrophage-specific genes.

A proposed role of Mitf and related transcription factors in osteoclast development (18, 27) tempted us to investigate a possible role of the MiT transcription factor family in macrophages that share a common precursor with osteoclasts (2). The PCR-based approach, which was designed to identify new and already known members of the MiT subfamily in macrophages, resulted in several interesting observations. In general, transcripts for all known MiT members could be identified in macrophages and OLCs. The presence of TFEB in osteoclasts confirms recent findings of Weilbaecher et al. (18), who identified TFEB as a dimerization partner of Mitf in these cells. Although the PCR-based approach was able to identify the mRNA of MiT members in macrophages and OLCs, it does not allow quantitative statements about transcript levels or extent of protein expression. In the case of cocultured OLCs, the detected fragments could conceivably originate from 5–10% residual primary osteoblasts that may contaminate the OLC-RNA preparation. Although no TFEC expression and only low levels of TFEB expression were detectable in RNA from primary osteoblasts (data not shown), we cannot rule out that their expression is induced in coculture with osteoclast precursors. However, these results suggest that TFEB or TFEC might also be possible dimerization partners for Mitf and TFEB in osteoclasts and certainly in macrophages. Interestingly, transcripts for TF3 and TFEB could also be detected in the melanoma cell line B16, which was used as a positive control for the expression of Mitf. This observation suggests that mature melanocytes may be able to express other MiT factors together with Mitf.

The previously undescribed murine homologue of TFEC appears restricted to macrophage-type cells, and this observation became the major focus of this study. Cloning and sequence analysis of mTFEC revealed a very similar structure and close homology with rat TFEC (22). The sequence structure of the human homologue TFEC-L is less homologous and includes an exon encoding an acidic activation domain that corresponds to a transcriptional activation domain of subfamily members TFEB and Mitf (23). Transient transfection studies revealed that hTFEC-L could slightly activate, although much less than Mitf or TFEB, or inhibit transactivation of different reporters, depending on the cell lines used (23). Based on sequence comparison, the properties of mTFEC probably resemble those of the rat homologue, which also

**FIGURE 6.** PU.1 binding to Ets motifs of the TFEC promoter in EMSA. A, rPU.1 protein and the labeled SV40 PU-box oligonucleotide were used in competition tests with unlabeled etbox oligonucleotides 1–6. B, EMSA of rPU.1 with individual etbox boxes compared with SV40 PU box. C, EMSA of RAW264.7 nuclear proteins with labeled etbox-6 oligonucleotide. PU.1 was competed by cold etbox-6 itself and by the SV40 PU box. The anti-PU.1 serum significantly shifted the gel and positions of free probe and shifted PU.1 are indicated in each case.

**FIGURE 7.** PU.1 and Ets-2 transactivate the TFEC promoter in fibroblasts. The fibroblast cell line NIH3T3 was cotransfected with the TFEC-promoter construct and expression plasmids of PU.1 and Ets-2, as described in Materials and Methods. Values represent the luciferase activity relative to the empty control vector pECE and are the mean ± SD obtained from three independent experiments.

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**FIGURE 8.** Highlight major characteristics found in the region of transcriptional initiation of a range of well-characterized myeloid-specific promoters. The aligned sequences generally contain multiple Ets-like recognition motifs. In many cases, these purine-rich sequences have been shown to be strictly necessary for basal promoter activity. The comparison also indicates another common, yet uncharacterized, motif (CCAGTG) that can be found in many myeloid promoters. A weak Ets-like binding motif (CAGGAA) that is essential for the activity of the c-fms promoter (29) and the promoter of PU.1 (38) itself is also present in the TFEC promoter at a similar position.
With the analysis of a growing number of macrophage-specific genes, it has become clear that their 5′-proximal promoter regions share several features that distinguish them from other genes. A typical macrophage-type promoter appears to lack TATA boxes, consensus initiator sequences, or GC-rich regions found in housekeeping genes, and instead contains multiple purine-rich sequence motifs that are recognized by transcription factors of the Ets family (14, 29, 38, 42–44, 51, 53–55). We cloned and sequenced a 600-bp region of the proximal TFEC promoter and found exactly six motifs that are recognized by transcription factors of the Ets family. The typical absence of TATA boxes or other conventional initiator sequences and the ability of PU.1 to bind directly to TFIID or the retinoblastoma gene product (56) suggest an alternative, macrophage-specific mechanism for transcriptional initiation that might involve interactions of different Ets family transcription factors and a myeloid-specific initiator element such as the CAGGAA sequence in c-fms, c-fes, PU.1, and TFEC.

Further studies will be needed to determine a functional role for mTFEC in macrophages. The published observations on rTFEC and hTFEC-L suggest that mTFEC would most likely act as a transcriptional repressor of other coexpressed MiT family members (22, 23). In all macrophage cell lines studied to date, TFEC was coexpressed with other subfamily members, and in certain macrophage cell types (e.g., BMM, TGE-PM), all four members, TFE3, TFEB, Mitf, and TFEC, can be detected. In theory, this would allow the formation of many different heterodimer combinations that could, on one hand, have individual properties, but, on the other hand, may also have overlapping functions. In this context, TFEC could be important in balancing the actions of coexpressed transactivators Mitf, TFE3, and TFEB at certain stages of development or in special macrophage subpopulations. It will be of interest to determine whether the relative levels of these four factors are regulated by agents that modulate macrophage differentiation.

Clearly, further studies are required to fully understand the function of MiT transcription factors in osteoclasts or macrophages. These studies may include knockout mutations of all members and their combinatorial analysis. Other important issues include the regulation of MiT-protein expression in macrophages and osteoclasts and, equally important, the identification of transcriptional targets in osteoclasts or macrophages that are currently unknown.

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FIGURE 8. Comparison of proximal promoter regions of several macrophage-restricted genes. The sequence of promoter regions of indicated genes was aligned using the CCAGNG motif that was found in proximal promoters of many myeloid-restricted genes. Published transcription initiation sites are indicated by arrows, and Ets-like GGAA motifs on either strand are marked as grey spheres. Black spheres indicate the position of motifs that greatly reduce myeloid-specific activity when mutated.
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