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# Genetic and Physical Interactions between *Microphthalmia* Transcription Factor and PU.1 Are Necessary for Osteoclast Gene Expression and Differentiation*

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**The *microphthalmia* transcription factor (MITF), a basic-helix-loop-helix zipper factor, regulates distinct target genes in several cell types. We hypothesized that interaction with the Ets family factor PU.1, whose expression is limited to hematopoietic cells, might be necessary for activation of target genes like tartrate-resistant acid phosphatase (TRAP) in osteoclasts. Several lines of evidence were consistent with this model. The combination of MITF and PU.1 synergistically activated the TRAP promoter in transient assays. This activation was dependent on intact binding sites for both factors in the TRAP promoter. MITF and PU.1 physically interacted when coexpressed in COS cells or *in vitro* when purified recombinant proteins were studied. The minimal regions of MITF and PU.1 required for the interaction were the basic-helix-loop-helix zipper domain and the Ets DNA binding domain, respectively. Significantly, mice heterozygous for both the mutant *mi* allele and a PU.1 null allele developed osteopetrosis early in life which resolved with age. The size and number of osteoclasts were not altered in the double heterozygous mutant mice, indicating that the defect lies in mature osteoclast function. Taken in total, the results afford an example of how lineage-specific gene regulation can be achieved by the combinatorial action of two broadly expressed transcription factors.**

The MITF[1] gene encodes a basic-helix-loop-helix zipper (bHLH-zip) protein highly related to the TFE3, TFEB, and TFEC gene products (1–4). Mutations in the human MITF gene result in the genetic diseases Waardenburg's syndrome 2A and Tietz syndrome (5, 6). In the rat, homozygous deletion of the MITF locus causes microphthalmia and pigment defects as well as an osteopetrosis that resolves with age (7, 8). At least 18 different mutant alleles at the MITF locus have been characterized in the mouse, and these alleles affect, to varying extents, differentiation of melanocytes, pigmented retinal epithelial cells, mast cells, and osteoclasts (9–11). Thus, MITF can selectively affect gene expression and differentiation of developmentally unrelated types of cells. A major interest of our laboratories is in understanding how MITF selectively activates target genes in osteoclasts as opposed to other cell types where the factor is expressed.

Osteoclasts differentiate from a myeloid progenitor to become mature, multinuclear cells capable of resorbing bone (12). Mice homozygous for the mutant *mi* allele develop severe osteopetrosis caused by a failure of mononuclear precursors to mature into multinuclear osteoclasts capable of bone resorption (13, 14). Bone marrow transplantation experiments demonstrate that the *mi* mutation acts in a cell-autonomous manner (15, 16), and *in situ* hybridization studies confirm that MITF is expressed in osteoclasts beginning at the earliest stages of endochondrial ossification of long bones (17). Understanding how MITF regulates gene expression in osteoclasts may provide insights into the molecular mechanisms that control terminal differentiation of this specialized cell type.

MITF and the related TFE factors form heterodimers and homodimers that bind to DNA sequences related to the E-box motif (CANNTG) shared by many helix-loop-helix transcription factors. In cell types affected by MITF mutations, MITF acts by binding to a *cis*-acting element, TCATGTG, located in the proximal promoters of lineage-specific genes. For example, genes encoding tyrosinase (melanocytes) and tartrate-resistant acid phosphatase (TRAP) (osteoclasts) require this *cis*-element for cell type-specific regulation (17, 18). These data indicate that MITF expression alone is likely insufficient to account for the regulation of selective targets in the different cell types. One hypothesis to account for MITF action is that in each cell type, MITF acts in concert with a unique combination of transcription factors to affect expression of target genes.

Expression of the Ets family transcription factor PU.1 distinguishes osteoclasts from melanocytes. PU.1 expression is limited to hematopoietic lineages and is necessary for differentiation of a number of cell types, in particular B-cells, macrophages, and osteoclasts (19, 20). Osteoclast differentiation fails at a very early stage in mice bearing a targeted disruption of the PU.1 gene, but PU.1 is expressed at all stages of osteoclast differentiation in wild-type cells, including in mature multinuclear cells (21). In addition, direct interaction between MITF and PU.1 *in vitro* has been detected (22).

In this report we present molecular, biochemical, and genetic evidence showing that PU.1 and MITF physically and function-

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[1] The abbreviations used are: MITF, *microphthalmia* transcription factor; bHLH-zip, basic-helix-loop-helix zipper; TRAP, tartrate-resistant acid phosphatase; PCR, polymerase chain reaction; GFP, green fluorescent protein; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; EMSA, electrophoretic mobility shift assay; RANKL, receptor activator of NF-κB ligand.

ally interact. We conclude that the interaction with PU.1 is necessary for MITF to affect osteoclast differentiation and gene expression.

## EXPERIMENTAL PROCEDURES

*Cell Culture and Transfections*—Cells were maintained in Dulbecco's modified Eagle's medium containing 4 mM L-glutamine, 50 $\mu$g/ml penicillin, 50 $\mu$g/ml streptomycin, and 5% newborn calf serum (NIH 3T3 and COS-7) or 10% fetal bovine serum (RAW264.7) at 37 °C in 7% $CO_2$. Differentiation of RAW264.7 cells was optimized using Dulbecco's modified Eagle's medium and 10% fetal bovine serum supplemented with 50 $\mu$g/ml ascorbic acid (Sigma), 20 ng/ml soluble recombinant human RANKL/osteoclast differentiation factor (PeproTech, Inc.), and $10^4$ units/ml recombinant human colony-stimulating factor-1 (a gift from Chiron Corp., Emeryville, CA). For flow cytometry cells were grown over a period of 5 days, changing the medium on day 2 and day 4.

NIH 3T3 cells were transfected using the calcium phosphate procedure as described previously (23). COS-7 cells were transfected using Superfect transfection reagent (Qiagen). Cells were plated at $2 \times 10^6$ cells in 100-mm dishes 24 h before the transfections. Cells were transfected with Superfect reagent as described by the manufacturer. Cells were harvested 24 h after transfection. RAW264.7 cells were transfected by electroporation as described previously (24).

Expression vectors for PU.1 and MITF and the TRAP luciferase reporter have been described previously (17, 25). Site-directed point mutations of PU.1 and MITF binding sites, indicated in Fig. 1, were introduced by PCR (17). The S36T enhanced green fluorescent protein (GFP) reporter gene-derived pEGFP-1 (CLONTECH) was fused to the TRAP proximal promoter (from −620 to +3, relative to the ATG codon).

*Flow Cytometry of Stably Transfected Cell GFP Reporter Gene Constructs*—Under low light conditions, cells were suspended in phosphate-buffered saline and incubated for 10 min at room temperature. Resuspended cells were analyzed for GFP expression using a FACSCalibur flow cytometer (Becton Dickinson). Data analysis was performed by analyzing 10,000 events for each assay using the CELLQuest software package (Becton Dickinson). The fluorescence intensity was divided into four regions, M1–4. M1 was set to encompass the background level of green autofluorescence exhibited by 99.9% of untransfected control cells, and M2, M3, and M4 represented logarithmic increments of fluorescence intensity (100-fold, 1,000-fold, and 10,000-fold above background, respectively). All events with fluorescence intensity greater than the M1 region were accepted as cellular GFP fluorescence events. The data are presented as follows.

$$\%\ \text{GFP-expressing cells} = \frac{(\text{M4})}{(\text{M2} + \text{M3} + \text{M4})} \qquad \text{(Eq. 1)}$$

*Protein Interaction Assays*—COS-7 cells were metabolically labeled with [$^{35}$S]methionine for 3 h. Cells were lysed for 30 min at 4 °C in a lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 6 mM $MgCl_2$, 1% Nonidet P-40) containing protease (10 $\mu$g/ml aprotinin, 10 $\mu$g/ml leupeptin, 100 $\mu$g/ml phenylmethylsulfonyl fluoride) and phosphatase (1 mM EGTA, 10 mM NaF, 1 mM tetrasodium pyrophosphate, 0.1 mM $\beta$-glycerophosphate, 1 mM $NaVO_3$) inhibitors. Cell lysates were centrifuged, precleared with 20 $\mu$l of protein G Gamma Bind Plus Sepharose beads (Amersham Pharmacia Biotech) for 45 min at 4 °C, and incubated with monoclonal anti-HA antibody (Babco) or polyclonal anti-MITF serum (raised against amino acids 1–111) overnight. Beads were precipitated and washed four times with lysis buffer and once with a low salt buffer (50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 6 mM $MgCl_2$, 1% Nonidet P-40). Samples were resolved on SDS-PAGE and analyzed by autoradiography.

The various DNA constructs encoding bacterial fusion proteins were generated by PCR amplification of PU.1 and MITF cDNAs with PCR primers defining the boundaries shown in Fig. 5. Both GST vectors (pGEX, Amersham Pharmacia Biotech) and $His_6$-tagged vectors (pET15b and pET32, Novagen) were used to make fusion proteins. Recombinant fusion proteins were purified by affinity chromatography following protocols supplied by the vector manufacturers. 1 $\mu$g of the recombinant GST or GST fusion bait protein was incubated with 20-$\mu$l glutathione-Sepharose beads (Amersham Pharmacia Biotech) in binding buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5% Nonidet P-40) in 300 $\mu$l for 1 h at 4 °C. 1 $\mu$g of the $His_6$-tagged test protein was then added to the reaction and incubated for another 2 h at 4 °C. Beads were precipitated and washed five times with binding buffer. Reaction products were resolved on SDS-PAGE and analyzed by Western blotting using anti-$His_6$ antibody (Santa Cruz).

*Electrophoretic Mobility Shift Assay*—EMSAs were performed as described previously using $His_6$-tagged versions of MITF and PU.1 (17). The sense strand oligonucleotide, representing the mouse TRAP proximal sequences, was CAGTTCTGGGGAAGTCCAGTGCTCACATGACCCA. The core of the PU.1 site (GGA to TTA) or of the E-box (CACAGT to CTCGAG) was mutated singly or in combination. The sense stands of the TRAP promoter and SV40 enhancer oligonucleotides used in Fig. 1*C* were CCAGTTCTGGGGAAGTCCAGTGCTC and CTGAAAGAGGAACTTGGTTAGGTA, respectively.

*Mice and Radiological/Histological Analyses*—The *mi* mutation was maintained on a B6C3Fe hybrid background as originally obtained from Jackson Laboratory (Bar Harbor, ME). The PU.1 knockout model was generously provided by Dr. Harinder Singh (University of Chicago) and was supplied in the 127J background (26). Mice heterozygous for both PU.1 and *mi* alleles were produced and then crossed to supply all combinations of the two alleles. The PU.1 null allele was genotyped by PCR as described (27). The *mi* allele was genotyped by PCR with primers that allowed the 3-base pair difference between wild-type and *mi* allele to be distinguished by gel electrophoresis. There was a 100% correspondence between eye pigment phenotype and genotype determined by this PCR method.

Newborn animals were euthanized and fixed in 4% paraformaldehyde in phosphate-buffered saline for 48 h at 4 °C, then transferred to 70% ethanol. Radiological analysis was performed on Faxitron x-ray machine (Hewlett Packard model 43855A) for 1–2 min at 35–45 kV using X-Omat V film (Kodak). Procedures for histological staining have been described previously (28).

For TRAP histochemical staining newborn femurs were embedded in plastic resin using a JB-4 embedding kit (Polysciences, Inc.) as described by manufacturer, and 5-$\mu$m sections were cut using the LKB Ultratome (LKB Instruments Inc., Rockville, MD). Samples were stained for TRAP activity using a leukocyte acid phosphatase kit (Sigma) as described by the manufacturer.

## RESULTS

*PU.1 Regulates the TRAP Promoter through a Conserved GGA Binding Site*—The proximal promoter of the mouse TRAP gene contains a conserved binding site for MITF which is necessary for the transcription of the gene during terminal osteoclast differentiation (17). A putative binding site for the Ets family transcription factor PU.1 (GGAA) is located around 10 base pairs upstream of the MITF binding site in the human, mouse, and pig TRAP genes (Fig. 1*A*). EMSAs were performed with this site using purified recombinant PU.1 (Fig. 1*B*). These experiments demonstrated that PU.1 specifically recognized the TRAP promoter probe containing the wild-type binding site, but not a probe with a mutated binding site (Fig. 1*B*, *lanes 1* and *2*). Additionally, PU.1 binding was competed efficiently by increasing amounts of unlabeled TRAP competitor DNA representing the wild-type binding site, but not the mutant site (Fig. 1*B*, *lanes 3–8 versus 9–14*).

To confirm that this site is bound by native PU.1, we examined RAW264.7 macrophage nuclear extracts by EMSA (Fig. 1*C*). PU.1 is the only macrophage nuclear protein that recognizes the high affinity PU.1 site from SV40 (25). When the TRAP sequence was used in EMSA a complex was detected which comigrated with the SV40 PU-box complex (Fig. 1*C*, *lanes 1* and *2*). Addition of anti-PU.1 polyclonal antibody, but not preimmune serum, could block formation of this complex (Fig. 1*C*, *lane 4 versus lane 3*). By comparison with the SV40 PU-box, the TRAP site was a substantially lower affinity site as judged by relative cold competition (Fig. 1*C*, *lanes 6–9* compared with *lanes 10–13*).

The ability of PU.1 to transactivate the TRAP promoter through this binding site was examined using transient transfection assays (Fig. 2*A*). In NIH 3T3 cells, which do not express PU.1, cotransfection of a PU.1 expression vector was able to activate the TRAP promoter by 4-fold (Fig. 2*A*, *left panel*). When the conserved binding site was mutated, activation by PU.1 was abrogated (Fig. 2*A*, *left panel*). Similar results were seen when transient assays were performed in RAW264.7 cells that express endogenous PU.1, and basal activity of the TRAP

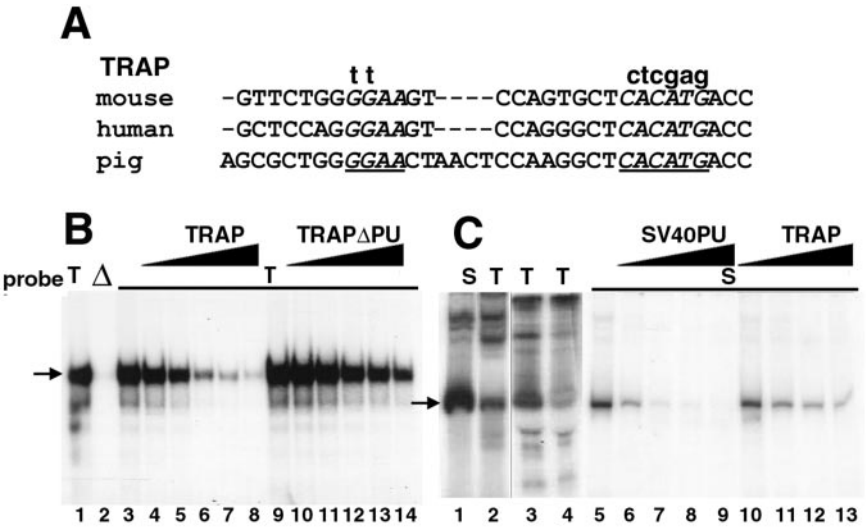


FIG. 1. **PU.1 binds to the conserved GGAA sequence on the TRAP promoter.** *Panel A*, sequences of the TRAP promoter from mouse, human, and pig contain a conserved PU.1 binding site (GGAA) upstream of the conserved E-box (CACATG). The mutations introduced into the PU.1 binding site and the E-box are shown in *small type* above the sequences. *Panel B*, 100 ng of the recombinant $His_6$-thioredoxin-PU.1 fusion protein was incubated with $^{32}P$ end-labeled wild-type (*T*) or mutant (GGAA to TTAA, Δ) TRAP promoter. The formation of the complex was competed with increasing amounts (2-, 5-, 10-, 50-, 100-, 200-fold molar excess, *lanes 3–8*, respectively) of cold wild-type probe (TRAP) but not with the same amounts of cold mutant probe (TRAPΔPU, *lanes 9–14*). The *arrow* indicates the PU.1·TRAP oligonucleotide complex. *Panel C*, RAW264.7 nuclear extracts were incubated with $^{32}P$ end-labeled TRAP (*T*) or SV40 enhancer (*S*) oligonucleotides. The effect of adding preimmune rabbit serum (*lane 3*) or anti-PU.1 antiserum (*lane 4*) to PU.1·TRAP complex formation is shown. Note that this antibody blocks complex formation rather than causing a band supershift (25). The formation of the SV40·PU.1 complex was competed with increasing amounts of the cold SV40 enhancer probe (SV40PU, 0, 5-, 25-, 50-, and 100-fold molar excess, *lanes 5–9*, respectively) or cold TRAP promoter probe (*TRAP*, same amounts of competitor, *lanes 10–13*). The *arrow* shows the PU.1-containing complex.

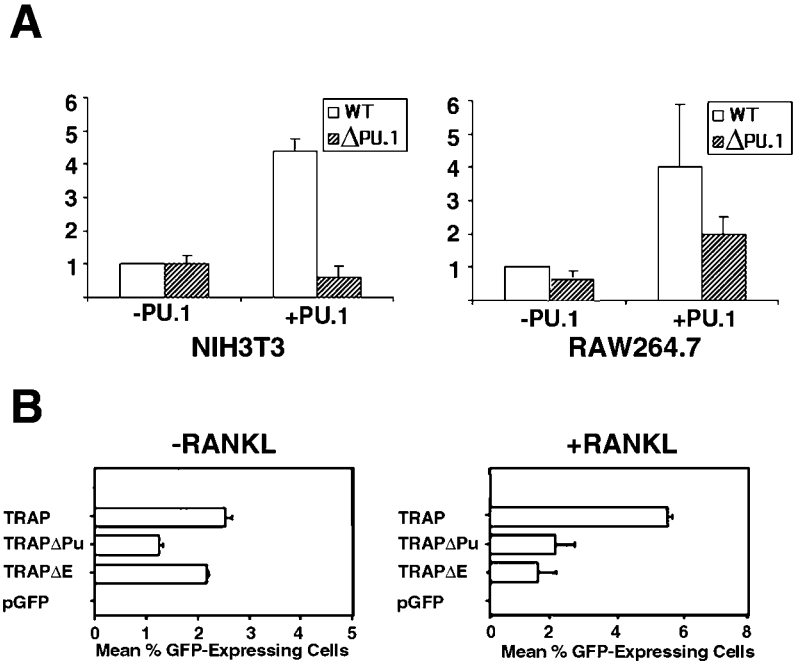


FIG. 2. **PU.1 transactivates the TRAP proximal promoter.** *Panel A*, NIH 3T3 or RAW264.7 cells (as indicated) were transfected with 5 μg of either TRAP wild-type (*WT*) luciferase reporter or the TRAP promoter with the mutated PU.1 site (GGAA to TTAA, Δ*PU.1*). The promoter-reporter constructs were transfected either alone (−*PU.1*, 0.5 μg or 2 μg of empty expression vector for 3T3 or RAW264.7, respectively) or together with PU.1-expressing vector (+*PU.1*, 0.5 μg or 2 μg of expression vector for 3T3 or RAW264.7, respectively). Promoter activity was expressed as relative luciferase units relative to the wild-type basal promoter activity (set to a value of 1). The results of four independent experiments performed in duplicate are represented, and the *error bars* indicate the standard deviation. *Panel B*, RAW264.7 cells were stably transfected with the indicated wild-type and mutated TRAP reporter constructs (either PU.1 or E-box sites mutated), and pooled clones were grown without RANKL or in the presence of RANKL for 5 days, as indicated. The GFP plasmid without promoter was included as a control. GFP expression was determined by flow cytometry (see "Experimental Procedures"). The graph represents the percentage of cells exhibiting high fluorescence ($10^3$-$10^4$ higher than background fluorescence) among all cells with GFP fluorescence. The average of three experiments is shown with *error bars* indicating the standard deviation.

promoter was decreased about 2-fold in this cell type as well (Fig. 2*A*, *right panel*).

Incubation of RAW264.7 cells with colony-stimulating factor-1 and RANKL can promote the differentiation of osteoclast-like cells (29). We used this *in vitro* differentiation system to examine the role of the conserved PU.1 binding site in regulat-

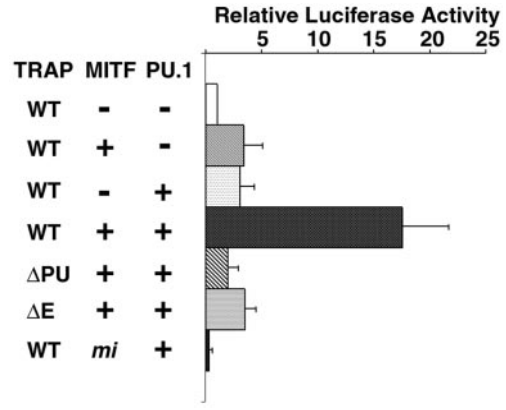


FIG. 3. **MITF and PU.1 collaborate to activate the TRAP promoter.** NIH 3T3 cells were transfected with the luciferase reporter (5 $\mu$g) driven by either the wild-type TRAP promoter (*WT*), or the TRAP promoter with mutations either in the E-box (CACATG to CTCGAG, Δ*E*) or in the PU.1 binding site (GGAA to TTAA, Δ*PU*). The promoter-reporter constructs were transfected either alone (with empty expression vector) or together with vectors expressing 0.5 $\mu$g of PU.1, 3 $\mu$g of MITF, or the combination of 0.5 $\mu$g of PU.1 and 3 $\mu$g of MITF. Promoter activity was expressed as the relative luciferase units with basal activity set to 1 as above. The results of four independent experiments, each performed in duplicate, are presented, with *error bars* indicating the standard deviation.

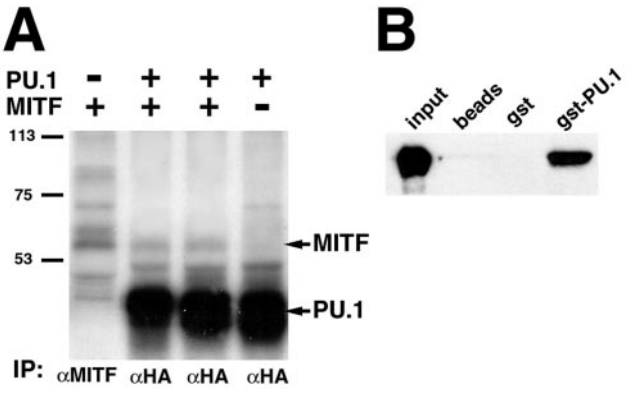


FIG. 4. **Physical interaction between MITF and PU.1.** *Panel A*, COS-7 cells were transiently transfected with expression vector for HA-PU.1, MITF (see "Experimental Procedures"), or both together. Cells were metabolically labeled with [$^{35}$S]methionine, and immunoprecipitation (*IP*) reactions were performed using antibodies against the HA tag or MITF. Reaction products were resolved on 8% SDS-PAGE and analyzed by autoradiography. *Panel B*, 1 $\mu$g of recombinant $His_6$-MITF was incubated with 1 $\mu$g of recombinant GST or GST-PU.1. After incubation with glutathione-Sepharose beads (or Sepharose beads alone) and washing, bound material was resolved on 8% SDS-PAGE and analyzed by Western blot using antibodies against the $His_6$ tag.

ing TRAP expression during differentiation of RAW264.7 cells (Fig. 2*B*). RAW264.7 cells were stably transfected with either a wild-type TRAP promoter linked to GFP or promoters with the PU.1 binding site or MITF binding site mutated. Pooled clones containing each construct were selected and subsequently induced to differentiate for 5 days with colony-stimulating factor-1 and soluble RANKL. Following this treatment, about 10% of cells in RAW264.7 cultures were multinuclear osteoclast-like cells (data not shown). Flow cytometry was used to study GFP expression in aspirated cells from these cultures (see "Experimental Procedures"). The analysis indicated that the percentage of highly fluorescent GFP cells increased ~3-fold following treatment of cultures with RANKL compared with untreated cultures. Mutation of the PU.1 site in the proximal promoter reduced GFP reporter activity in RANKL-treated cells by 3-fold to the level found in untreated cultures (Fig. 2*B*, *TRAP*Δ*PU.1*, *right panel*). The PU.1 mutation also had a smaller effect on reporter activity in untreated RAW264.7 cells (Fig. 2*B*, *left panel*). As a positive control, we reproduced the observation that mutation of the MITF site resulted in significantly reduced TRAP promoter activity following osteoclast differentiation (Fig. 2*B*, *TRAP*Δ*E*, *+RANKL*, 17).

*MITF and PU.1 Cooperate in Activation of TRAP Reporter Genes*—To test the hypothesis that interaction with PU.1 might account for the ability of MITF to regulate gene expression in osteoclasts, the combined ability of the two factors to activate the TRAP promoter was studied using transient transfection assays. Expression vectors for PU.1 or MITF alone activated the TRAP reporter 4–5-fold, but the combination of the two factors activated the TRAP reporter 20-fold (Fig. 3). When either the E-box or the PU.1 binding site in the TRAP promoter was mutated, the combination of MITF and PU.1 failed to superactivate the reporter (Fig. 3). The MITF/*mi* mutation, which encodes a protein lacking the ability to bind to the conserved TRAP E-box (17), failed to superactivate the TRAP reporter in combination with PU.1 (Fig. 3). These results imply a functional interaction between MITF and PU.1 which is dependent on DNA binding of both transcription factors to the TRAP promoter.

*Physical Interactions between MITF and PU.1*—Sato and co-workers (22) have demonstrated previously that MITF and PU.1 can physically interact *in vitro* when bound to DNA. To confirm and extend these results, biochemical assays were used to study the physical interaction of the two factors in the absence of DNA (Figs. 4 and 5). Coimmunoprecipitation of PU.1 and MITF in COS-7 cells was studied (Fig. 4*A*). Expression vectors encoding HA-tagged PU.1 and wild-type MITF were cotransfected into COS-7 cells. HA-PU.1 was immunoprecipitated from cells metabolically labeled with [$^{35}$S]methionine, and the immunoprecipitates were examined by denaturing gel electrophoresis. The experiments demonstrated that a protein with the same mobility as MITF was coprecipitated with PU.1 (Fig. 4*A*, *arrow*). This protein was absent from precipitates obtained either from cells expressing only HA-PU.1 (Fig. 4*A*) or in mock-transfected cells that expressed neither protein (data not shown). In addition, if preimmune serum was used, or if the primary antibody was not included in the immunoprecipitation reaction, neither MITF nor PU.1 was detected (data not shown).

In a complementary set of experiments, full-length recombinant proteins were expressed in *Escherichia coli*. $His_6$-tagged MITF and GST-PU.1 were separately expressed and purified. GST-PU.1 bound to glutathione-Sepharose beads was used in "pull-down" assays with $His_6$-tagged MITF (Fig. 4*B*). These experiments demonstrated that $His_6$-tagged MITF could bind to GST-PU.1 Sepharose beads but not to GST-alone beads (Fig. 4*B*).

To map the domain of MITF involved in the interaction with PU.1, a series of MITF deletion mutations fused to GST was tested in pull-down assays using $His_6$-thioredoxin-tagged PU.1 (Fig. 5). The fragment of MITF containing amino acids 199–298, which included the bHLH-zip domains (Fig. 5*A*), was able to interact with PU.1 as efficiently as the full-length protein (Fig. 5*C*, *top panel*). The protein encoded by the MITF/*mi* allele was also bound as efficiently as wild-type protein (Fig. 5*C*, *top panel*, *last two lanes*). Neither the N-terminal region including the basic domain (amino acids 1–217) nor the C-terminal portion that included the HLH-zip domain (amino acids 217–419) was able to bind PU.1 above the background level observed with GST alone (Fig. 5*C*, *top panel*). In control experiments the MITF deletion proteins did not bind to $His_6$-thioredoxin alone (Fig. 5*C*, *bottom panel*). Therefore, both basic and HLH-zip domains of MITF are necessary for efficient PU.1 binding.

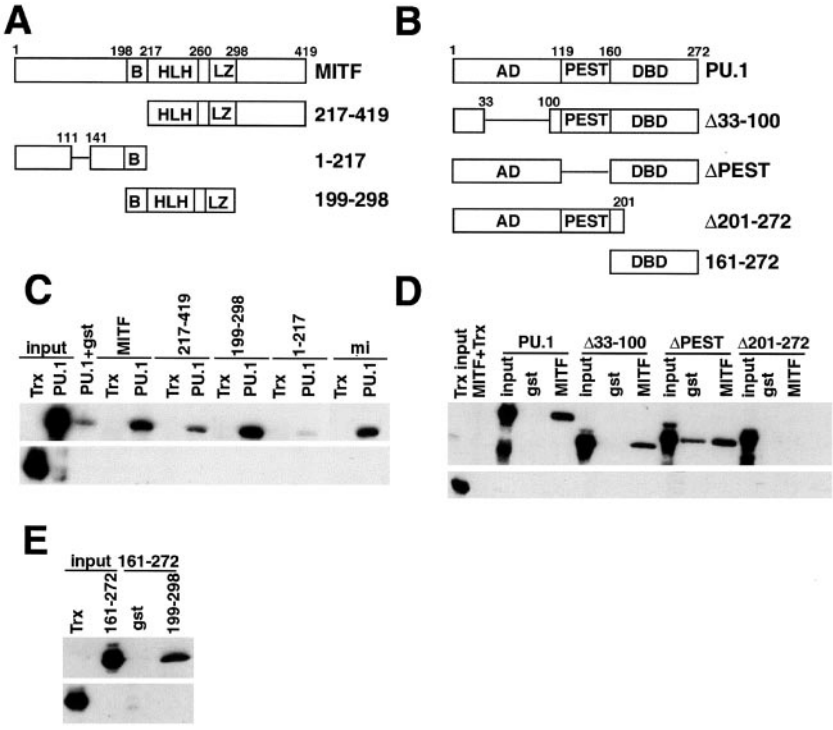


FIG. 5. **Identification of MITF and PU.1 interacting domains.** *Panel A*, diagram of MITF, indicating protein domains expressed as GST fusion proteins in *E. coli.* The position of the *mi* mutation, a deletion of one of four critical arginine residues in the basic region, is indicated (*arrow*). *LZ*, leucine zipper. *Panel B*, diagram of PU.1 indicating protein domains expressed as $His_6$-thioredoxin fusion proteins in *E. coli.* In *panels C–E*, the binding reactions with recombinant proteins were incubated with glutathione-Sepharose beads (or Sepharose beads alone) and washed; bound material was eluted and resolved on 10% SDS-PAGE and analyzed by Western blotting using antibodies against $His_6$ tag (Santa Cruz Biotechnology). *DBD*, DNA binding domain; *AD*-activation domain. *Panel C*, 1 μg of recombinant $His_6$-thioredoxin-PU.1 (*PU.1*) or $His_6$-thioredoxin (*Trx*) was incubated with 1 μg of recombinant GST or GST-MITF fusion proteins, as indicated. MITF-*mi* represents full-length MITF protein with the 3-base deletion that defines the *mi* allele. *Panel D*, 1 μg of recombinant PU.1 or PU.1 deletion mutants (Fig. 5*B*) fused to $His_6$-thioredoxin or $His_6$-thioredoxin alone (*Trx*) was incubated with 1 μg of recombinant GST or MITF bHLH-zip fused to GST (amino acids 199–298). *Panel E*, 1 μg of PU.1 DNA binding domain (amino acids 161–272) fused to $His_6$-thioredoxin or $His_6$-thioredoxin alone (*Trx*) was incubated with 1 μg of recombinant GST or GST fused to the bHLH-zip domain of MITF (amino acids 199–298).

PU.1 is a 272-amino acid protein that consists of the N-terminal activation domain, PEST domain, and C-terminal DNA binding domain (Fig. 5*B*; 20). To map the MITF interacting domain of PU.1 a series of deletions fused to $His_6$-thioredoxin was constructed and tested in pull-down binding assays using GST-MITF (amino acids 199–298) as bait (Fig. 5*D*). Recombinant PU.1 proteins lacking either the activation domain (Δ33–100) or the PEST domain (ΔPEST) were able to interact with MITF, but the protein without the DNA binding domain (Δ201–272) lost the ability to bind MITF (Fig. 5*D*).

To confirm the minimal regions of MITF and PU.1 required for the interaction, the *in vitro* binding assay was performed using amino acids 199–298 of MITF fused to GST (Fig. 5*A*) and amino acids 161–272 of PU.1 fused to $His_6$-thioredoxin (Fig. 5*B*). These experiments demonstrated that the DNA binding domain of PU.1 was able to bind GST-MITF 199–298 fusion protein, but not GST (Fig. 5*E*). Inclusion of ethidium bromide in these reactions had no effect on the pull-down results, consistent with the conclusion that interaction between free proteins in solution, and not protein bacterial DNA complexes, is being measured in these assays (data not shown).

*PU.1 and MITF Do Not Bind in a Cooperative Fashion to the TRAP Promoter*—A possible explanation for the cooperation between MITF and PU.1 is that the two factors bind in a cooperative fashion to target genes like TRAP. To investigate the mechanism of cooperation between PU.1 and MITF, we performed an EMSA using a TRAP promoter probe containing binding sites for both PU.1 and MITF. When both PU.1 and MITF were present in the reaction, we observed that a new band with lower electrophoretic mobility was detected, representing a ternary complex containing PU.1, MITF, and TRAP promoter sequences.

Mutation of the binding site for either transcription factor abolished the formation of the complex containing both MITF and PU.1 (Fig. 6*A*). Cold competition experiments to measure off time rates of the complexes were also performed (Fig. 6*B*). Consistent with the data presented above, the PU.1 complex had a very rapid off rate of less than 30 s. The MITF complex was very stable, with little competition observed even after 30 min. The complex containing both factors behaved the same as the PU.1 complex, with a very rapid off rate (Fig. 6*B*). Taken together, the data indicate that the presence of MITF, which binds stably and with high affinity, does not alter the low affinity binding of PU.1 to the adjacent site.

*Genetic Interaction between MITF and PU.1*—Ultimately, the relevance of interactions between MITF and PU.1 can only be assessed by examining interactions at the genetic level in intact animals, where both the target cell population and the levels of expression of the gene products are appropriate. Mice heterozygous for either the *mi* allele (MITF/*mi*) or for a PU.1 knockout allele do not develop an apparent bone phenotype. To test for the possibility of genetic interaction between MITF and PU.1 we crossed heterozygous MITF/*mi* mice with heterozygous PU.1+/− mice to obtain double heterozygous MITF/*mi*/PU.1+/− animals. The resulting mice were analyzed for bone density and morphology by radiological techniques and hematoxylin and eosin staining (Figs. 7, *A* and *B*, respectively). Approximately 25% of the double heterozygous MITF/*mi*/PU.1+/− mice were clearly osteopetrotic compared with 0% of PU.1+/− and 5% of MITF/*mi* single heterozygotes, respectively

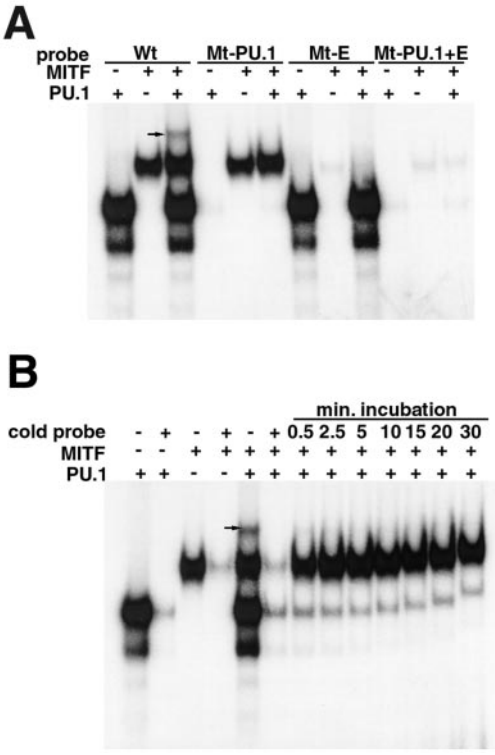


FIG. 6. **MITF and PU.1 form a ternary complex on the TRAP promoter.** *Panel A*, MITF and PU.1 binding to the TRAP promoter was not cooperative *in vitro.* 100 ng of recombinant $His_6$-thioredoxin-PU.1 fusion protein and 100 ng of recombinant $His_6$-MITF protein were incubated with $^{32}P$ end-labeled wild-type (*Wt*), GGAA to TTAA mutant (*Mt-PU.1*), CACATG to CTCGAG mutant (*Mt-E*), or double mutant (*Mt-PU.1+E*) TRAP promoter oligonucleotides (see "Experimental Procedures"). The location of the MITF·PU.1·pTRAP complex is indicated with an *arrow*. *Panel B*, 100 ng of recombinant $His_6$-thioredoxin-PU.1 fusion protein and/or 100 ng of recombinant $His_6$-MITF protein was incubated with $^{32}P$ end-labeled wild-type TRAP promoter oligonucleotides (see "Experimental Procedures"). A 200-fold molar excess of the cold TRAP oligonucleotide was added to the reaction mix either before the addition of the labeled probe (+) and incubated for 3 min, or after the addition of the labeled probe and incubated for 0.5, 2.5, 5, 10, 15, 20, or 30 min, as indicated. The location of the MITF·PU.1·TRAP complex is indicated with an *arrow*.

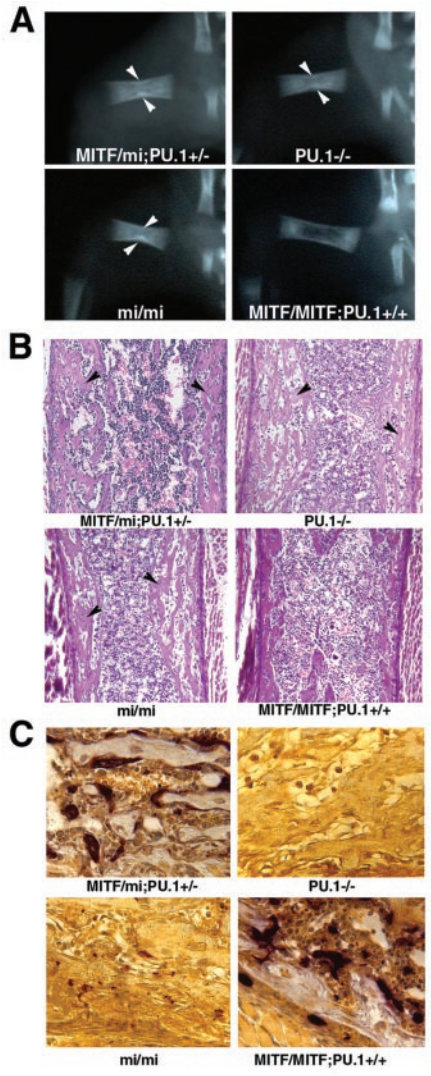


FIG. 7. **Osteopetrotic phenotype of double heterozygous MITF/*mi*/PU.1+/−.** *Panel A*, radiological analyses of the femur from mice of the genotype indicated. High density areas of the unresorbed calcified tissue in double heterozygous PU.1−/− and *mi*/*mi* samples are indicated by *arrows*. *Panel B*, histological staining of the femur sections from mice of the genotypes indicated. Eosin-stained unresorbed calcified tissue in double heterozygous PU.1−/− and *mi*/*mi* samples is indicated by *arrows*. Samples were observed at a magnification of ×100. *Panel C*, osteoclasts from femur sections from mice of the indicated genotypes were detected by TRAP histochemical staining *in situ*. Samples were observed at a magnification of ×400.

(Table I). Although the osteopetrosis in the double heterozygote mice was detected readily at birth, it resolved with age so that eruption of teeth occurred, and by sexual maturity bone density as assayed by radiography appeared normal (data not shown).

Surprisingly, we found that mice of genotype PU.1−/− also survived to birth. The PU.1−/− strain used in this report has previously been found to die *in utero* because of severe hematological abnormalities (26). The number of mice surviving was lower than predicted for mice that of genotype PU.1−/−;*mi*/*mi* and PU.1−/−;MITF/*mi* (10 and 50% of expected numbers, respectively, Table I). The PU.1−/− mice that survived exhibited osteopetrosis (Fig. 7).

Osteoclasts in sections from animals of the various genotypes were visualized by histochemical staining for TRAP activity (Fig. 7*C*). The results showed that in *mi*/*mi* mice numerous, small osteoclasts were detected, whereas in PU.1−/− mice no TRAP-positive osteoclasts were detected, in accordance with previous reports for both types of models (14, 21). In the double heterozygote mutant mice that developed osteopetrosis, the morphology and number of osteoclasts were not significantly different from those in the wild-type (Fig. 7*C*).

## DISCUSSION

*MITF and PU.1 Interact during Osteoclast Differentiation*—One possible mechanism that could account for the selective regulation of osteoclast target genes is that MITF might cooperate with a hematopoietic cell-restricted factor in this cell type. Results from both transfection studies and from *in vitro* biochemical assays support the hypothesis that the Ets family factor PU.1 and MITF act together to regulate the mature osteoclast marker gene, TRAP. Consistent with the biochemical data, combining mutant alleles for MITF and PU.1 in mice demonstrated an interaction between these two genes *in vivo* during osteoclast differentiation.

Osteopetrosis was detected early in life in mice heterozygous for a PU.1 knock-out allele and the *mi* allele. The morphology and number of osteoclasts appeared normal in bone sections examined in the compound heterozygous mice, indicating that the defect occurred during the later stages of osteoclast differentiation or in fully mature osteoclasts. The similar phenotypes of mice with targeted deletions of two known MITF target genes, TRAP and cathepsin K, support a role for both MITF and PU.1 in mature osteoclasts (17, 30–32). Multinuclear os-

TABLE I
*Genotypes and phenotypes of mice carrying mi and PU.1 null alleles*

| PU.1[a] genotype | MITF[a] genotype | Actual no. of mice | Predicted[b] no. of mice | No. of op mice | % op mice |
|---|---|---|---|---|---|
| +/+ | MITF/MITF | 19 (12.8%) | 9.25 (6.25%) | 0 | 0 |
| +/− | MITF/MITF | 29 (19.5%) | 18.5 (12.5%) | 0 | 0 |
| +/− | MITF/*mi* | 42 (28.3%) | 37 (25%) | 10 | 24 |
| +/+ | MITF/*mi* | 21 (14.1%) | 18.5 (12.5%) | 1 | 5 |
| +/+ | *mi*/*mi* | 5 (3.3%) | 9.25 (6.25%) | 5 | 100 |
| +/− | *mi*/*mi* | 16 (10.8%) | 18.5 (12.5%) | 16 | 100 |
| −/− | *mi*/*mi* | 1 (0.6%) | 9.25 (6.25%) | 1 | 100 |
| −/− | MITF/MITF | 6 (4.5%) | 9.25 (6.25%) | 6 | 100 |
| −/− | MITF/*mi* | 9 (6.1%) | 18.5 (12.5%) | 9 | 100 |

[a] MITF/*mi*/PU.1+/− double heterozygous mice were crossed, and offspring were genotyped as described under "Experimental Procedures."
[b] Predicted numbers are based on a Mendelian inheritance of unlinked genes and a total of 148 mice analyzed.

teoclasts are present in both TRAP and cathepsin K knock-out models, but these osteoclasts are not fully functional, and mild osteosclerotic disease occurs in the mice (31, 32). The inability to regulate expression of these MITF target genes properly could account for the phenotype reported here (17, 30). It is worth noting that the cathepsin K promoter region shown to be necessary for MITF regulation also contains consensus PU.1 recognition sequences (30).

Approximately 25% of double PU.1/*mi* heterozygotes developed the osteopetrotic phenotype. The incomplete penetrance of the phenotype may reflect genetic redundancy in osteoclast differentiation and partial compensation for the loss of PU.1 and MITF function by other genes involved in this process. An alternate possibility is that these results may reflect that the experiments were performed in an outbred genetic background resulting from the F2 cross of the unrelated MITF/*mi* and PU.1+/− lines, and that genetic modifiers specific for either PU.1 or MITF alter the penetrance of the observed phenotype. The potential role of genetic background is supported by the unexpected survival of PU.1−/− mice until birth. Two PU.1 knock-out models have been generated, and different phenotypes have been observed (26, 33). In the mice generated by Maki and colleagues (33), mice survive postpartum but succumb rapidly to microbial infections unless maintained with antibiotics or rescued by bone marrow transplantation. In the mutant allele used in the experiments reported here, lethality was observed at days 16–18 of embryonic development because of severe hematological abnormalities (26).

The less penetrant PU.1 null phenotype has been attributed to incomplete functional disruption of the PU.1 gene (19), but the effect of the genetic background suggested by the results presented here has not been reported previously. We found that ~50% of PU.1 homozygous null mice were born. These mice died in the 1st week of life, similar to the other PU.1 knock-out model (33), and these mice also developed severe osteopetrosis typified by a lack of osteoclasts in developing bone, the same phenotype reported for the other PU.1 knock-out model (21). The results argue for the existence of genetic modifiers that reduce the penetrance of a PU.1 null mutation, with MITF as one potential candidate modifier. Fewer PU.1 null mice survived to birth than predicted when they were homozygous for the *mi* allele, and we have demonstrated previously that MITF is expressed prior to PU.1 in embryonic macrophages (34).

The related transcription factors TFE3 and TFEC, both expressed in osteoclasts (4), could be potential modifiers of MITF. Based on biochemical data, it has been hypothesized that TFE3 may modify the osteopetrotic phenotype observed in rats homozygous for the *microphthalmia blanc* allele, a recessive allele containing a large deletion of the rat MITF gene (8). The osteopetrotic phenotype of this rat resolves with age, similar to the phenotype of the double *mi*/PU.1 heterozygotes (7, 8). Similarly, other Ets family transcription factors expressed in the macrophage lineage (*e.g.* Ets-2, ELF-1, MEF, 35, 36), some of which can also *trans*-activate the TRAP promoter[2], could ameliorate the effects of the absence of PU.1. Additional biochemical and genetic analyses will be required to determine the role of these bHLH-ZIP and Ets factors as well as to identify other putative PU.1 and MITF partners in osteoclast differentiation.

*The Mechanism of MITF and PU.1 Collaboration*—PU.1 interactions with several different transcription factors have been documented (37). For example, PU.1 recruits the transcription factor PIP to DNA through direct physical interactions, an event necessary for activation of target genes in B-cells (38–40). These results led us to anticipate that MITF and PU.1 might bind cooperatively to DNA, but all evidence obtained here indicated that PU.1 and MITF bound independently to TRAP promoter sequences. Because MITF and PU.1 are coexpressed in both macrophages and osteoclasts, a mechanism based on cooperative DNA binding might lead to inappropriate expression of genes like TRAP in macrophages. Further, if the two factors bind to DNA independently, the weak TRAP PU.1 binding site may provide a mechanism by which TRAP promoter activity is sensitive to the increased PU.1 expression levels observed during osteoclast differentiation (21).

Binding sites for both PU.1 and MITF were required for superactivation of the TRAP promoter, indicating that a ternary complex with DNA might be requisite for functional interactions to occur. By contrast, the bHLH factor MyoD and its cofactor for regulation of muscle-specific genes, MEF2, require only a single binding site for either factor to allow synergistic activation of target genes (41). The active ternary complex formed when both factors bind to DNA may result in conformational changes in both proteins which allows for more efficient interaction with transcriptional coactivators. PU.1 and MITF have both been shown to be targets of signaling pathways in other cell types (39, 42, 43). Modification of either MITF or PU.1 by signaling pathways could also enhance the formation of the ternary complex and couple complex formation to signals triggering osteoclast differentiation.

Other mechanisms that depend on PU.1 and MITF interaction prior to DNA binding cannot be ruled out by our data. The overexpression of MITF/*mi* can lead to retention of PU.1 in the cytoplasm of transfected WEHI-3 cells (22). The formation of a PU.1·MITF complex in the cytoplasm and cotranslocation into the nucleus could ensure that the two factors are recruited together to promoters such as TRAP, effectively increasing the on rate for binding and the likelihood that both sites on the promoter are occupied. Such a mechanism could also be regulated by post-translational modification of either or both factors.

[2] A. I. Cassady and D. A. Hume, unpublished data.

In summary, we have shown that PU.1 and MITF cooperate to activate the process of osteoclast differentiation and transcriptional activation of the osteoclast-specific TRAP gene. These findings add to the increasing evidence that unique combinations of widely distributed transcriptional regulators like MITF and PU.1 can generate lineage-restricted gene expression.

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# Genetic and Physical Interactions between*Microphthalmia* Transcription Factor and PU.1 Are Necessary for Osteoclast Gene Expression and Differentiation

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