The mi locus of mice encodes a member of the basic helix-loop-helix leucine zipper (bHLH-Zip) protein family of transcription factors (hereafter called MITF, for mi transcription factor).1,2 Spontaneous chemical, radiation, and insertional mutageneses provide abundant mutant alleles at the mi locus,3,4 which are useful for the analysis of the relationship between the structure and function of MITF.5-8 The mutant allele that has been studied most intensively is mi. The mi/mi mice show depletion of pigment in both hair and eyes, microphthalmia, osteopetrosis, and deficient natural killer activity.5,4 In addition, the number of mast cells decreases and their phenotype is abnormal in mi/mi mice.9-15 Although most mast cells in the skin of normal (+/+ ) mice are stained with berberine sulfate that binds heparin proteoglycan, few mast cells are berberine sulfate+ in the skin of mi/mi mice.13,16-19 

Cultured mast cells (CMCs) derived from the spleen of mi/mi mice are deficient in the expression of various genes, such as the mouse mast cell protease (MMCP)-4,20 MMCP-5,21 MMCP-6,22 c-kit,23 p75 nerve growth factor receptor,24 granzyme B (Gr B),25 tryptophan hydroxylase (TPH),26 integrin α4 subunit,26 and α-melanocyte-stimulating hormone receptor genes.27 MITF encoded by the mi mutant allele (mi-MITF) deletes 1 of 4 consecutive arginines in the basic domain.1,6,7 The mi-MITF is defective in the DNA binding ability and the nuclear localization potential.28,29 The mi-MITF does not appear to transactivate target genes due to these abnormalities.20-27,29 

The tg is another mutant allele of the mi locus.1,30 The tg/tg mice possess the insertional mutation at the promoter region of mi gene and do not express any MITFs.1,31 The tg/tg and mi/mi mice share several phenotypic features, but the phenotypic abnormality of tg/tg mice is apparently mild compared with that of mi/mi mice. The transcription of c-kit, Gr B, and TPH genes was significantly reduced in mi/mi CMCs, but the reduction was moderate in tg/tg CMCs.32 This indicated that the presence of mi-MITF caused more severe abnormalities than the absence of normal (+) MITF. In addition to the loss of transactivation ability, the mi-MITF possesses an inhibitory effect on the transcription of some particular genes in mast cells.32 Because the tg is considered to be a null mutant allele, the tg/tg mice may be useful for evaluating the function of other mutant MITFs. When a homoygous mouse at a certain mi allele shows more severe phenotype than that of the tg/tg mouse, the MITF encoded by the mutant mi allele may possess an inhibitory function. When a homoygous mouse at another mutant mi allele shows a phenotype comparable to that of the tg/tg mouse, the MITF encoded by the mutant mi allele may not possess any functions.

MITF encoded by the mi mutant allele (mi-MITF) lacks the Zip domain because of a stop codon between HLH and Zip.6 To our
knowledge, the mi<sup>−</sup> is the only available mutant of genes encoding bHLH-Zip proteins lacking the Zip domain. In the present study, we compared the phenotype of mast cells of mi<sup>−/−</sup> mice with that of mi<sup>+/−</sup> or tg/tg mice to clarify the importance of the Zip domain of MITF for development of mast cells. The phenotype of mi<sup>−/−</sup> mast cells was similar to that of tg/tg mast cells rather than to that of mi<sup>+/−</sup> mast cells, indicating that the ce-MITF had no functions.

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**Materials and methods**

**Mice**

The original stock of C57BL/6-mi<sup>+/+</sup> mice was purchased from the Jackson Laboratory (Bar Harbor, ME) and was maintained in our laboratory by consecutive backcross with our own inbred C57BL/6 colony (more than 15 generations at the time of the present experiments). The original stock of Vγ9-Tg/tg mice, in which the mouse vasopressin–Escherichia coli β-galactosidase transgene was integrated at the 5′ flanking region of the mi gene, were kindly given by Dr H. Arnheiter (National Institutes of Health, Bethesda, MD). The integrated transgene was maintained by repeated backcrosses to our own inbred C57BL/6 colony (more than 10 generations at the time of the present experiment). The original stock of C57BL/6-mi<sup>−/−</sup> mice were maintained in the laboratory of Dr L. Lamoreux (Texas A&M University, College Station, TX). The C57BL/6-mi<sup>−/−</sup> mice were crossed to our own inbred C57BL/6 colony, and the mi<sup>−/−</sup> mice were selected by sequencing of the MITF gene. Female and male heterozygous mi<sup>+/+</sup>, mi<sup>−/−</sup>, or mi<sup>−/−</sup> mi<sup>−/−</sup> mice were crossed together, and the resulting homozygous mi<sup>+/+</sup>, mi<sup>−/−</sup>, or mi<sup>−/−</sup> mi<sup>−/−</sup> mice were selected by their white coat color. C57BL/6-mi<sup>−/−</sup> mice were used in our laboratory as a control.

**Cells**

Pokeweed mitogen–stimulated spleen cell–conditioned medium (PWM-SCM) was prepared according to the method described by Nakahata et al. Mice of mi<sup>+/−</sup>, mi<sup>−/−</sup>, mi<sup>−/−</sup>, or mi<sup>−/−</sup> were used at 2 to 3 weeks of age to obtain CMCs. Mice were killed by decapitation after ether anesthesia, and spleens were removed. Spleen cells were cultured in α-minimal essential medium (α-MEM; ICN Biomedicals, Costa Mesa, CA) supplemented with 10% PWM-SCM and 10% fetal calf serum (FCS; Nippon Bio-supp Center, Tokyo, Japan). Half of the medium was replaced every 5 days. Cells derived from the spleen of each mutant genotype reached 10<sup>7</sup> in number within 4 weeks. More than 95% of cells contained alcin blue<sup>−</sup> granules and were considered to be CMCs 4 weeks after initiation of the culture. The NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Flow Laboratories, Irvine, UK) supplemented with 10% FCS. The P815 cells were maintained in α-MEM supplemented with 10% FCS.

**Staining and counting of mast cells**

Mice 20 days of age were killed by decapitation after ether anesthesia. Pieces of dorsal skin were removed, smoothed onto a piece of the filter paper to keep them flat, fixed in Carnoy’s solution, and embedded in paraffin. Sections of skin pieces were stained with alcian blue or with paraffin. The technique of in situ hybridization was described in detail. To obtain the MMCP-4, MMCP-5, MMCP-6, and mast cell carboxypeptidase A (MC-CPA) probes, single-stranded complementary DNA (cDNA) was generated from total RNA extracted from CMCs of +/+ mouse origin by lithium chloride-urea method. The specific cDNA of proteases was then amplified with specific primers for each protease by polymerase chain reaction (PCR). The cDNAs were subcloned into the EcoRV site of pBluescript KS– plasmid (pBS; Stratagene, La Jolla, CA) that contains T3 and T7 promoters to promote the probes.

After hybridization with the antisense probe, the cells possessing signals that were stronger than those obtained with the sense probe were considered to be messenger RNA<sup>+</sup> (mRNA<sup>−</sup>). We counted the number of mRNA<sup>−</sup> cells per centimeter of skin. In the adjacent section, the number of alcin blue<sup>−</sup> cells per centimeter of skin was counted. Then, the proportion of various protease mRNA<sup>−</sup> cells to alcin blue<sup>−</sup> cells was calculated.

**Northern blot analysis**

Each RNA sample was prepared from 1 × 10<sup>7</sup> CMCs by the lithium chloride-urea method. Northern blot analysis was performed using c-kit, MMCP-4, MMCP-5, MMCP-6, MC-CPA, Gr B, TP-β<sub>2</sub>, and glyceraldehyde-3-phosphate dehydrogenase ( GAPDH) cDNAs labeled with [α-<sup>32</sup>P]deoxyctydine triphosphate (370 MBq/mL; NEN Life Science Products, Boston, MA) by random oligonucleotide priming. After hybridization at 42°C, blots were washed to a final stringency of 0.2 × SSC (1 × SSC is 150 mM NaCl and 15 mM trisodium citrate, pH 7.4) and subjected to autoradiography.

**Cytotoxicity assay**

Mast cell cytotoxicity was measured using a 51Cr release assay according to the procedure described by Bissonnette and Befus. As a positive control, spleen cells were freshly prepared from 8-week-old +/+ mice. The target was YAC-1 cells, which were obtained from American Type Culture Collection (Bethesda, MD). The cells were maintained in α-MEM supplemented with 10% FCS. CMCs derived from +/+ and mi<sup>−/−</sup> and tg/tg mice and spleen cells of +/+ mice were washed, suspended in α-MEM supplemented with 10% FCS, and distributed at different concentrations (0.5, 1.0, and 2.5 × 10<sup>5</sup> cells) in triplicate into 96-well microtiter plates with round bottoms. YAC-1 cells (5 × 10<sup>4</sup>) were labeled with 3.7 MBq of [<sup>51</sup>Cr]Na<sub>2</sub>CrO<sub>4</sub> (Amersham-Pharmacia Biotech, Amersham Place, UK) for 2 hours, washed 3 times, and resuspended in α-MEM supplemented with 10% FCS. Labeled YAC-1 cells (1.0 × 10<sup>5</sup>) were mixed with various numbers of CMCs in a total volume of 200 μL/well. Plates were incubated at 37°C for 18 hours in a CO<sub>2</sub> incubator and spun at 150g for 10 minutes, and the radioactivity was determined in 100 μL samples of cell-free supernatants. The radioactivity released in the well containing YAC-1 cells alone was designated spontaneous release (SR). Total 51Cr release (TR) was measured by adding 0.01% Triton X-100 to the well containing YAC-1 cells alone. The percentage of specific 51Cr release was calculated using the following formula: (cpm in the presence of CMCs − SR)/(TR − SR) × 100.

**Concentration of serotonin**

The concentration of serotonin was measured using high performance liquid chromatography (HPLC) with electrochemical detection. Briefly, CMCs were collected, washed with phosphate-buffered saline (PBS), counted, and sonicated for 20 seconds in a sonicator (Tomy, Tokyo, Japan) in 1 mL ice-cold 3% perchloric acid containing 5 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM sodium metabisulfite. The homogenate was centrifuged at 10 000g for 15 minutes at 4°C, and the supernatant was applied directly to the HPLC column. The concentration of serotonin per 1.0 × 10<sup>5</sup> cells was calculated.

**Electrophoretic gel mobility shift assay (EGMSA)**

The production of the fusion protein containing glutathione-S-transferase (GST) and MITF was described previously. To examine the DNA binding ability of MITF, an oligonucleotide that is a part of MMCP-6 promoter was...
used as a probe. The sequence of the oligonucleotide is 5′-TTGGTGGGGA-CACATGTTACAGGA (the sequence recognized by +MITF is underlined). The oligonucleotide was labeled with α-32P-dideoxyctydine triphosphate by filling 5′ overhangs and used as probes of EGMSA. DNA-binding assays were performed in a 20 μL reaction mixture containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 75 mM KCl, 1 mM dithiothreitol (DTT), 4% Ficoll 400, 50 ng poly(dI-dC), 25 ng labeled DNA probe, and 3.5 μg GST-MITF fusion protein. After the incubation at room temperature for 15 minutes, the reaction mixture was subjected to electrophoresis at 14 V/cm at 4°C on a 5% polyacrylamide gel in 0.25 Tris-borate-EDTA (TBE) buffer (1 × TBE is 90 mM Tris-HCl, 64.6 mM boric acid, and 2.5 mM EDTA, pH 8.3). The polyacrylamide gels were dried on Whatman 3MM chromatography paper and subjected to autoradiography.

Construction of expression plasmids and immunocytochemistry

The pBS containing the whole coding region of +MITF, MITF, or mi-MITF was constructed in our laboratory (hereafter called pBS +MITF, pBS-s+MITF, and pBS-mi-MITF, respectively). To generate the Myc-tagged MITF construct, we subcloned the Smal-HincII fragment of pBS +MITF, pBS-ce-MITF, or pBS-mi-MITF into the SalI site of the CS2 +MT expression vector that provides 6 copies of the Myc epitope tag at the N-terminal end of the protein (a gift from Dr I. Matsumura, Osaka University, Osaka, Japan). The resultant chimeric gene was subcloned into pEF-BOS expression vector kindly provided by Dr S. Nagata (Osaka University, Osaka, Japan). The expression plasmid was transfected into NIH3T3 cells, and the overexpressed MITF protein was detected by anti-Myc antibody as described previously. Briefly, the cells were fixed with 100% methanol, permeabilized by treatment with 0.2% Triton X-100 in PBS, and incubated with the mouse monoclonal anti-Myc antibody (9E10; Pharmingen, San Diego, CA). Immunoreacted cells were detected by direct immunofluorescence with goat antimeumouse immunoglobulin G antibody conjugated with fluorescein isothiocyanate (MBL, Nagoya, Japan).

ce-MITF cDNA containing the nuclear localization

The nuclear localization signal (NLS) of SV40 large-T antigen (PKKKRKV) was inserted into the N-terminus of ce-MITF by PCR. The amplified product was verified by sequencing and cloned into CS2 +MT expression vector. Then, the resultant chimeric gene was subcloned into pEF-BOS expression vector. The subcellular localization of the ce-MITF cDNA containing the nuclear localization signal of pEF-BOS was examined by immunocytochemistry as described above.

Immunoblotting for the nuclear and cytoplasmic extracts

Nuclear and cytoplasmic extracts of NIH3T3 cells transfected with expression plasmid of Myc-tagged +MITF, ce-MITF, or mi-MITF were prepared as described before. Briefly, transfected cells were washed with PBS twice and resuspended in ice-cold buffer containing 10 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Then, Nonidet P-40 was added to a final concentration of 0.1%. After vigorous vortexing, the homogenate was centrifuged at 3000 rpm for 3 minutes. The supernatant was used as the cytoplasmic fraction. The pellet was resuspended in ice-cold buffer containing 400 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH 7.4), and 1 mM PMSF, kept on ice for 1 hour, and centrifuged at 15 000 rpm. The supernatant was used as the nuclear fraction. Samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Immobilon P, Millipore, Bedford, MA). The blots were incubated with 5% skim milk in Tris-buffered saline (20 mM Tris-HCl [pH 7.4], 150 mM NaCl). Then, the blots were incubated with Tris-buffered saline containing 5% skim milk with the anti-Myc antibody. The membrane was incubated with peroxidase-conjugated goat antimouse immunoglobulin G antibody, and the immune complexes were visualized with Western blot chemiluminescence reagent (NEN Life Science Products).

Transient cotransfection assay

The reporter plasmid that contained Gr B promoter starting from nt −910 (+1 shows a transcription initiation site) was previously reported. As the expression plasmids, the Smal-HincII fragment of ce-MITF, or pBS-mi-MITF, was introduced into the blunted Xhol site of pEF-BOS, and the PCR-amplified fragment of ce-MITF with the NLS of SV40 large-T antigen was also cloned into the blunted Xhol site of pEF-BOS. A total of 10 μg of a reporter, 2 μg of an expression plasmid, and 3 μg of an expression vector containing β-galactosidase gene were cotransfected into P815 cells by electroporation. The expression vector containing β-galactosidase gene was used as an internal control. The cells were harvested 48 hours after the transfection and lysed with 0.1 M potassium phosphate buffer (pH 7.4) containing 1% Triton X-100. Soluble extracts were then assayed for luciferase activity with a luminometer LB960F (Berthold, Wildbad, Germany) and for β-galactosidase activity. The luciferase activity was normalized by the β-galactosidase activity and total protein concentration according to the method described by Yasumoto et al. The normalized value was expressed as the relative luciferase activity.

Results

The number of mast cells was examined in the skin of mi−/mi mice. Histologic sections of the skin pieces of mi−/mi mice were stained with alcian blue. The number of mast cells in the mi−/mi mice decreased to one third that of the control +/+ mice and was comparable to that of mi/mi and tg/tg mice (Table 1). In +/+ mice, most of skin mast cells were berberine sulfate+; indicating that they contained heparin. The proportion of berberine sulfate+ mast cells in the skin of mi−/mi mice was comparable to that of +/+ mice and was also comparable to that of tg/tg mice (Table 1). In contrast, the proportion of berberine sulfate+ mast cells was 3% in the skin of mi/mi mice, as reported previously.

The expression of mast cell–specific protease genes was analyzed by in situ hybridization in mast cells of the skin. The proportion of MMCP-4 mRNA+ and MMCP-6 mRNA+ mast cells decreased remarkably in the skin of mi−/mi mice, but that of MMCP-5 mRNA+ and MC-CPA mRNA+ mast cells did not (Table 2). No significant differences were detectable in the expression of mast cell proteases among skin mast cells of mi−/mi and mi/mi mice.

The expression of genes that had been demonstrated to be affected by MITF was examined in CMCs derived from the spleen of mi−/mi, tg/tg, and mi/mi mice using Northern blot. The amount of c-kit mRNA reduced in mi/mi CMCs, as reported previously. The amount of c-kit mRNA of mi−/mi CMCs was comparable to that of tg/tg CMCs and was intermediate between the amount of +/+ CMCs and that of mi/mi CMCs (Figure 1). The amounts of MMCP-4, MMCP-5, and MMCP-6 mRNAs in mi−/mi CMCs reduced to the levels comparable to those of mi/mi and tg/tg CMCs (Figure 1). In contrast to the reduced expression of c-kit mRNA, the expression of the genes encoding collagen I and II was comparable to that of +/+ CMCs in mi−/mi CMCs and was increased in mi/mi CMCs.

Table 1. Number of mast cells and proportion of berberine sulfate+ cells to alcian blue+ cells in the skin of +/+ , mi/mi, mi−/mi*, and tg/tg 20-day-old mice

<table>
<thead>
<tr>
<th>Genotype of mice</th>
<th>No. alcian blue+ cells per cm of skin</th>
<th>Proportion of berberine sulfate+ to alcian blue+ cells, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>426 ± 29</td>
<td>95 ± 5</td>
</tr>
<tr>
<td>mi−/mi</td>
<td>142 ± 26†</td>
<td>83 ± 5</td>
</tr>
<tr>
<td>mi/mi</td>
<td>125 ± 20†</td>
<td>3 ± 1†</td>
</tr>
<tr>
<td>tg/tg</td>
<td>168 ± 31†</td>
<td>64 ± 6</td>
</tr>
</tbody>
</table>

* Mean ± SE of 5 mice.
† P < .01 compared with the value of +/+ mice by t test.
MMCP-5 mRNA in mi/ce CMCs, the proportion of MMCP-5 mRNA+ mast cells was not reduced in the skin of mi/ce mice (Table 2). We previously reported that the addition of stem cell factor (SCF) significantly increased the amount of MMCP-5 mRNA in mi/ce CMCs and speculated that SCF synthesized by skin fibroblasts may induce the MMCP-5 expression in mi/ce skin mast cells. The different pattern of MMCP-5 expression between CMCs and skin mast cells of mi/ce, mg/tg, and mg/mg mice may be attributable to the different concentration of SCF surrounding mast cells.

The amount of Gr B mRNA reduced in mi/ce CMCs to a level comparable to that of mg/tg CMCs, but their magnitude of reduction was significantly smaller than that of mi/mg CMCs (Figure 1). We compared the cytotoxic activity of various CMCs, because Gr B mediates the cytotoxic activity of mast cells against YAC-1 cells. CMCs of +/+, mg/mg, mg/mg, and mg/tg genotype were cultured together with 51Cr-labeled YAC-1 cells, and the 51Cr release from YAC-1 cells was measured after 18 hours. At an effectortarget (E:T) ratio of 50, neither +/+, mg/mg, mg/mg, nor mg/tg CMCs showed any cytotoxic activity (Table 3). At an increased E:T ratio of 100 or 250, a remarkable cytotoxic activity of mi/mg CMCs was detected as in the case of +/+ or mg/tg CMCs, but no cytotoxic activity was observed in mg/mg CMCs (Table 3).

The amount of TPH mRNA also reduced in mi/mg CMCs to a level comparable to that of mg/tg CMCs, but the magnitude of reduction was significantly smaller than that of mg/mg CMCs (Figure 1). The serotonin content of various CMCs was compared, because TPH is the rate-limiting enzyme of the serotonin synthesis. The serotonin content of mi/mg CMCs was comparable to that of mg/tg CMCs. Both values were smaller than the value of +/+ CMCs but were larger than the value of mg/mg CMCs (Table 4).

We then examined functions of ce-MITF. First, the DNA binding ability of ce-MITF was examined by EMSA. A part of the MMCP-6 promoter containing the MITF binding motif, CACATG, was used as a probe. The specific binding of ce-MITF was not detectable as in the case of mi-MITF (Figure 2). Second, the subcellular localization of ce-MITF was examined by immunocytochemistry. The expression vector that contained +/MITF, ce-MITF, or mi-MITF cDNA downstream from the sequence of Myc-epitope was transfected into NIH3T3 cells. The localization of MITF was detected with anti-Myc antibody. A strong signal was detected only in the nucleus of the NIH3T3 cells overexpressing Myc-tagged +/MITF (Figure 3A). In contrast, signals were detected in both nucleus and cytoplasm of the NIH3T3 cells overexpressing Myc-tagged ce-MITF (Figure 3A). Signals were detected in both nucleus and cytoplasm as well in the NIH3T3 cells overexpressing Myc-tagged mi-MITF (Figure 3A). When the modified ce-MITF possessing the NLS of SV40 large-T antigen

**Table 2.** Proportion of MMCP-4, MMCP-5, MMCP-6, and MC-CPA mRNA+ cells to alcin blue+ cells in the skin of +/+, mg/mg, mg/mg, and mg/tg 20-day-old mice

<table>
<thead>
<tr>
<th>Genotype of mice</th>
<th>Proportion of cells expressing mRNA of each protease to alcin blue+ cells, %*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MMCP-4</td>
</tr>
<tr>
<td>+/+</td>
<td>55 ± 4</td>
</tr>
<tr>
<td>mg/mg</td>
<td>5 ± 1†</td>
</tr>
<tr>
<td>mg/mg</td>
<td>6 ± 2†</td>
</tr>
<tr>
<td>mg/tg</td>
<td>5 ± 2†</td>
</tr>
</tbody>
</table>

* Mean ± SE of 5 mice.
† P < .01 compared with the value of +/+ mice by t test.

**Table 3.** Cytotoxic activity of +/+, mg/mg, mg/mg, and mg/tg CMCs to YAC-1 cells

<table>
<thead>
<tr>
<th>E:T ratio</th>
<th>+/+ CMCs</th>
<th>mg/mg CMCs</th>
<th>mg/mg CMCs</th>
<th>+/+ spleen CMCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>52.2 ± 3.8†</td>
</tr>
<tr>
<td>100</td>
<td>12.8 ± 0.2†</td>
<td>10.1 ± 0.3†</td>
<td>11.9 ± 0.6†</td>
<td>58.3 ± 5.2†</td>
</tr>
<tr>
<td>250</td>
<td>25.2 ± 2.4†</td>
<td>16.6 ± 2.3†</td>
<td>19.1 ± 1.6†</td>
<td>NE</td>
</tr>
</tbody>
</table>

E:T ratio is the ratio of CMCs or spleen cells to YAC-1 cells. NE indicates not examined.
* Mean ± SE of 3 experiments.
† P < .01 by t test compared with the value of mg/mg CMCs.

**Table 4.** Concentration of serotonin in +/+, mg/mg, mg/mg, and mg/tg CMCs

<table>
<thead>
<tr>
<th>Genotype of CMCs</th>
<th>Serotonin concentration (nmol/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>8.86 ± 0.73</td>
</tr>
<tr>
<td>mg/mg</td>
<td>1.32 ± 0.08†</td>
</tr>
<tr>
<td>mg/mg</td>
<td>5.02 ± 0.35†</td>
</tr>
<tr>
<td>mg/tg</td>
<td>6.24 ± 0.28†</td>
</tr>
</tbody>
</table>

* Mean ± SE of 3 experiments.
† P < .05 compared with the value of +/+ mice by t test.
‡ P < .05 compared with the value of mg/tg mice by t test.
cells transfected with Myc-tagged +MITF cDNA but not in the cytoplasmic fraction. In contrast, moderate signals were detected in both nuclear and cytoplasmic fractions of the NIH3T3 cells transfected with Myc-tagged ce-MITF or Myc-tagged mi-MITF cDNA (Figure 3B). The size of the immunoreactive protein in NIH3T3 cells transfected with Myc-tagged ce-MITF cDNA was smaller than that of NIH3T3 cells transfected with Myc-tagged +MITF cDNA or Myc-tagged mi-MITF cDNA because of the truncation of the C-terminal region of ce-MITF (Figure 3B).

We examined the effect of ce-MITF on the transactivation of the Gr B promoter using the transient cotransfection assay (Figure 4). The 5’ flanking sequence of the Gr B gene (nt −910 to +42) was cloned upstream of the luciferase gene. Three functional CANNTG motifs were present in this region.25 The luciferase construct was cotransfected into P815 cells with the expression plasmid containing no insert. The luciferase activity obtained by the expression of vector alone (Figure 4) was further examined the modified ce-MITF that possessed the NLS of SV40 large-T antigen. The luciferase activity obtained by the expression of the modified ce-MITF was comparable to the value obtained by the expression of original ce-MITF (Figure 4).

Discussion

We examined the importance of Zip domain of MITF by comparing the mast cell abnormalities of mi-mi/ce-mi mice with those of tg/tg and mi/mi mice. First, we examined the abnormalities of skin mast cells. In mi/mi mice, the number of skin mast cells decreased to a level comparable to that of mi/mi and tg/tg mice. Although the proportion of berberine sulfate+ mast cells decreased in mi/mi mice, the proportion was normal in mi/mi mice as in the case of tg/tg mice. The proportions of MMCP-4 mRNA+ and MMCP-6 mRNA skin mast cells reduced in mi/mi mice to levels comparable to those of tg/tg and mi/mi mice. The abnormalities of skin mast cells of mi/mi mice were similar to those of tg/tg mice rather than to those of mi/mi mice.

Next, we compared the phenotype of mi/mi CMCS to that of tg/tg and mi/mi CMCS. The amounts of MMCP-4, MMCP-5, and MMCP-6 mRNAs reduced in mi/mi CMCS to levels comparable to those of tg/tg and mi/mi CMCS. The amounts of c-kit, Gr B, and
TPH mRNAs in mi/mi CMCs were comparable to those of tg/tg CMCs and were intermediate between the amounts of +/+ and those of mi/mi CMCs. The phenotype of mi/mi CMCs was similar to that of tg/tg CMCs rather than to that of mi/mi CMCs. The level of c-kit mRNA expression in mi/mi and tg/tg CMCs was higher than that of mi/mi CMCs. In contrast, numbers of mast cells in the skin of mi/mi and tg/tg mice were comparable to those of mi/mi mice. The expression level of c-kit in CMCs was not necessarily proportional to the number of mast cells in the skin. The mechanisms remain to be clarified.

There was a possibility that the decreased mast cell number in the skin of 20-day-old mi/mi, mi/mi/mi, and tg/tg mice might be a consequence of delayed homing or development of the mutant mast cells. If the decrease in 20-day-old mice was due to the delay of homing or development, the number of mast cells may be corrected in older mice. We examined the number of mast cells in the skin of approximately 60-day-old tg/tg mice, but the number of mast cells was comparable to that of 20-day-old tg/tg mice (unpublished data). We considered that the homing or development of mast cells was completed in 20-day-old mutant mice.

CMCs of mi/mi genotype killed YAC-1 cells as effectively as +/+ or tg/tg CMCs, but the cytotoxic activity of mi/mi CMCs was deficient. This was partly consistent with the expression level of Gr B mRNA demonstrated by the Northern analysis. Probably the expression level of Gr B observed in mi/mi and tg/tg CMCs may be enough for the cytotoxic activity. Serotonin contents of +/+ , mi/mi/mi, tg/tg, or mi/mi CMCs were well correlated with the expression levels of the TPH mRNA in mice of each genotype. Because we have not determined the amount of Gr B or TPH proteins, we were not able to indicate the direct correlation between mRNA and protein levels. Determination of the amounts of Gr B and TPH proteins will clarify this point.

We examined the function of ce-MITF by the luciferase assay using the Gr B promoter. As previously reported, the expression of ++MITF increased the activity of Gr B promoter significantly, whereas the expression of mi-MITF reduced it. The expression of ce-MITF showed a promoter activity comparable to the value obtained by the expression of vector alone, suggesting that the ce-MITF lacked not only the transactivation ability but also the inhibitory effect on transcription. This was consistent with the fact that the phenotype of mi/mi CMCs was similar to that of tg/tg mast cells.

The mi-MITF showing the inhibitory effect possessed the mutated basic domain and the intact Zip domain. Because the ce-MITF did not show such an inhibitory effect, the Zip domain may be necessary for the inhibitory effect of MITF. This was consistent with the result of Krylov et al that mutants of various bHLH-Zip proteins showing inhibitory effects possessed the abnormally basic domain and the normal Zip domain.

The effect of Zip domain on DNA binding ability has been reported in various bHLH-Zip proteins. Mutant upstream stimulatory factor (USF) and Max lacking the Zip domain bind DNA. On the other hand, TFE3 lacking Zip domain did not bind it. Fisher and his colleagues reported that the ce-MITF synthesized by reticulocyte lyastes did not bind DNA. Here, we obtained the same result using recombinant GST-ce-MITF fusion protein. The Zip domain of MITF and TFE3 appeared to be indispensable for DNA binding, whereas the Zip domain of USF and Max did not. The different attitude of Zip domain between MITF/TFE3 and USF/Max may be related to the fact that MITF forms a heterodimer with TFE3 but not with USF or Max.

The ce-MITF was detected both in the nucleus and cytoplasm as in the case of mi-MITF. Small molecules less than 50 kd are able to pass through the nuclear pore by passive diffusion. Because the molecular mass of monomeric Myc-tagged ce-MITF was approximately 50 kd, the monomeric form of ce-MITF may diffuse passively through the nuclear pore. The inability to dimerize and the reduction of the molecular mass due to truncation of Zip domain might cause passive diffusion of ce-MITF. The second explanation is that the Zip domain of MITF possesses an NLS. Recently, Nagoshi et al reported that the NLS located in the Zip domain of sterol regulatory element binding protein 2 (SREBP2), another bHLH-Zip protein. The truncation of Zip domain abolishes the nuclear localization of SREBP2. Further biochemical studies may clarify whether the NLS is present in the Zip domain of MITF.

There was a possibility that the abnormality of ce-MITF was simply due to its inability to translocate into the nucleus. To examine this possibility, we constructed the modified ce-MITF that possessed the NLS of SV40 large-T antigen. The expression of the modified ce-MITF did not show any promoter activities as that of the original ce-MITF. This indicated that the abnormality of ce-MITF was not due to a defect in the nuclear localization.

Taken together, the Zip domain was important for the function of MITF. The mi/mi/mi mice are useful for clarifying the function of MITF.

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Importance of leucine zipper domain of miRNA transcrip-
tion factor (MITF) for differentiation of mast cells demonstrated using mi ce /mi ce mutant mice of which MITF lacks the zipper domain

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