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Transcriptional activation of mouse mast cell protease-9 by microphthalmia-associated transcription factor

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Abstract

We explored transcriptional regulation of mouse mast cell protease-9 (mMCP-9), which is implicated in inflammation of the jejunum during helminth infections and tissue remodeling of the uterus during pregnancy. Transcription was positively regulated by microphthalmia-associated transcription factor (MITF), a member of the basic helix-loop-helix–leucine zipper family that binds to the E-box, a CANNTG sequence. The most significant segment for positive regulation by MITF was nt −183 to −177 of the mMCP-9 promoter, CATCATG, which bound MITF-M. In addition, not only other MITF isoforms but also TFE3, another member of the family, activated mMCP-9 transcription through this nucleotide sequence inserted one base within the E-box.

Keywords: Microphthalmia-associated transcription factor; Mouse mast cell protease-9; Basic helix-loop-helix leucine zipper; Transcription

Mouse mast cell proteases (mMCPs) are major components of the secretory granules of mature mast cells. At present nine mMCPs have been identified in a family of serine proteases and they are differentially expressed in tissue-specific populations of mast cells during differentiation and maturation. Based on their substrate specificities, mMCPs have been designated chymases or tryptases and play an important role in allergic and inflammatory reactions [1].

Microphthalmia-associated transcription factor (MITF) is a tissue-specific transcription factor predominantly expressed in mast cells, melanocytes, heart and skeletal muscle [2–4], and is a member of the basic helix-loop-helix–leucine zipper (bHLHLZ) family [2]. This transcription factor acts as a regulator of mast cell differentiation [5]. Although local growth factors and cytokines also affect expression of mMCPs [1], expression of a dominant-negative mutant MITF (*mi*-MITF) inhibited gene expression of mMCP-4 [6], -5 [7], -6 [8], and -7 [9], suggesting that MITF is absolutely necessary for gene expression of mMCPs.

Here, we report transcriptional regulation of mouse mast cell protease-9 (mMCP-9) gene. The present study revealed a positive regulation of mMCP-9 by MITF at the transcription level, and identified the most significant segment for the transcriptional activation by MITF in the 5′-flanking region of mMCP-9.

Materials and methods

Isolation of 5′-flanking region of mMCP-9 gene and construction of reporter plasmids. The isolation of 5′-flanking region of mMCP-9 gene was performed with the Mouse Genome Walker kit (Clontech) in accordance with the manufacturer's instructions. The isolated promoter region was cloned into pCR2.1 plasmids (Invitrogen). To construct reporter plasmids, a DNA fragment containing a promoter region and the first exon of the mMCP-9 gene (nt −779 to +32; nt +1 as the transcription initiation site) was cloned into a region upstream of the luciferase gene in pGL3-Basic vector (Promega). Subsequently, subclones containing progressive unidirectional deletions of the fragment were constructed using a deletion kit (TaKaRa). The reporter plasmids with point mutations were constructed by use of PCR techniques with

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mismatch sense and antisense primers. The products were verified by DNA sequencing.

cDNA constructs. Expression vectors pEF-BOS [10] containing the whole coding region of MITF-M, MITF-E or *mi*-MITF-M [3,11] were kindly provided by Drs. Y. Kitamura and E. Morii. Dr. C.M. Takemoto provided MITF-mc and TFE3 cDNAs [4]. MITF-M cDNA containing *Bgl*II and *Eco*RI restriction enzyme sites at the 5′- and 3′-ends, respectively, was subcloned into the vector pGEX-2T using the *Bam*HI and *Eco*RI sites.

Reporter assay. Luciferase assays were conducted as described previously [12]. HepG2 cells and MC/9 mast cells were obtained from RIKEN Cell Bank and ATCC, respectively. For transient transfection, HepG2 cells in 24-well plates were transfected with the indicated expression vectors, reporter construct, and a plasmid expressing β-galactosidase (pCMV-βGal) using polyfect transfection reagent (Qiagen). MC/9 cells were transfected by electroporation at 310 V and 950 μF using a Bio-Rad Gene Pulser II (Bio-Rad). Equal amounts of DNA were transfected in each experiment and adjusted with pcDNA1 and pcDNA3, and the cells were harvested 40 h after transfection. Luciferase activity was normalized to β-galactosidase activity.

Animals and cell culture. The original stock of C57BL/6-*mil*+ (*mil*/+) mice was purchased from the Jackson Laboratory (Bar Harbor, ME). Female and male *mil*/+ mice were crossed together and the resulting *mi*/*mi* mice were selected by their white coat color. Pokeweed mitogen-stimulated spleen cell-conditioned medium (PWM-SCM) was prepared according to the method described by Nakahata et al. [13]. Mice (C57BL/6-+/+ or *mi*/*mi*) were used at 2–3 weeks of age to obtain cultured mast cells (CMC). Mice were killed by decapitation and spleens were removed. Spleen cells were cultured in αMEM supplemented with 10% PWM-SCM and 10% fetal bovine serum. Half of the medium was replaced every 7 days. After 4 weeks of culture, more than 95% of cells were CMC. B16 melanoma cells, which were obtained from the Institute of Development, Aging and Cancer, Tohoku University, were cultured in DMEM with 10% fetal bovine serum.

RNA isolation and RT-PCR. RNA isolation from CMC and B16 cells and cDNA synthesis were conducted as described previously [14]. To examine gene transcripts of mMCP-9 and MITF isoforms, equal amounts of the reverse transcriptase reaction were individually subjected to PCR amplification for 35 cycles of 30 s at 94 °C, 30 s at 58 °C, and for 60 s at 72 °C after 5 min at 94 °C, followed by 8 min at 72 °C. PCR to detect the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH), was also conducted. PCR primers for G3PDH are described previously [14,15]. For mMCP-9 gene transcript (GenBank Accession No. AY007568), oligonucleotides coding for positions 1373–1395 and 1839–1814 were used as PCR primers. PCR primers for MITF-M, -mc, -H, and common MITF were as described by Takemoto et al. [4] and 5′-primer of MITF-E was as described by Oboki et al. [3]. The PCR products were separated on 2% agarose gels in 1× TAE buffer and visualized with ethidium bromide.

Electrophoresis mobility shift assay. GST or GST-MITF protein was expressed in *Escherichia coli* and purified with glutathione–Sepharose beads according to the manufacturer's protocol. An oligonucleotide probe corresponding to nt −190 to −170 of mMCP-9 promoter was end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP, followed by annealing and purification by gel-filtration (Sephadex G-25, Roche, Indianapolis, IN). Purified protein (2 μg) was pre-incubated for 20 min at 25 °C in binding buffer (10 mM Hepes, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT, 10% glycerol, 50 μg/ml poly(dI-dC), and 500 μg/ml bovine serum albumin) in the presence or absence of excess of a double-stranded competitor DNA. A radiolabeled DNA probe was then added and the incubation was continued for 10 min at 25 °C. The incubation mixture was loaded on a 5% non-denaturing polyacrylamide gel in 0.5× TBE buffer (45 mM Tris, 44 mM boric acid, and 1 mM EDTA) and electrophoresed for 2.5 h at 196 V. The gel was exposed to an imaging plate (Fuji, Tokyo, Japan).

Results

Transcriptional regulatory region of mMCP-9 gene

The 5′-flanking region of mMCP-9 gene was isolated from ICR Swiss mice (GenBank Accession No. AB075228). The region corresponded to nt 46686225–46687069 of chromosome 14. Transcriptional regulation of mMCP-9 gene was examined using luciferase-based reporter constructs. We tried reporter assays in primary cultured mast cells and mast cell lines, P-815 and IC-2, but the transfection efficiency was too low to detect luciferase and β-galactosidase expression reproducibly. Previous luciferase-based reporter assays revealed that mMCP-4 and -6 genes were positively regulated by MITF in NIH-3T3 cells and HMC-1 mast cells [4,6,8,16]. The present study used HepG2 cells, in which we obtained similar results on the transcriptional regulation of mMCP-4 and -6 (data not shown).

Several mMCPs are positively regulated by MITF [5]. MITF binds to the E-box, a CANNTG sequence, and this binding is necessary for transcriptional activation of the target genes [5,17]. The isolated 5′-flanking region has 3 CANNTG sequences (nt −556 to −551, nt −381 to −376, and nt −325 to −320). Therefore, we first predicted that some of the CATATG sequences are responsible for transcriptional activation of mMCP-9 gene. When a reporter construct containing nt −779 to +32 of mMCP-9 promoter was used, luciferase expression was increased as expected in response to MITF-M co-expression (Fig. 1). However, unexpectedly, reporter assays using deletion constructs showed that the regulatory region influenced by MITF-M spanned nt −247 to −90, in which there were no CANNTG sequences (Fig. 1). To specify the region responsible for transcriptional activation by MITF-M, we constructed further deletion mutants of the reporter genes. As a result, a region between nt −184 and −179 was critical for MITF-M-dependent transcriptional activation of mMCP-9 gene (Fig. 2A). The nucleotide sequence located between nt −184 and −176 is CATCATG (Fig. 2B) and is essentially the same as the CANNTG sequence with an extra base inserted. We assumed that this CANNNTG sequence is crucial for transcriptional activation by MITF-M. The reporter construct containing the mMCP-9 promoter starting from nt −192 responded to expression of MITF-M, but point mutations of TG at nt −178 and −177 to CT abolished responsiveness to MITF-M (Fig. 3), suggesting the importance of the CATCATG sequence for transcriptional activation of mMCP-9.

To examine whether MITF-M binds to the CATCATG sequence, electrophoresis mobility shift assay (EMSA) was performed using purified MITF protein. When an oligonucleotide spanning nt −190 to −170 of mMCP-9 promoter was used as a probe, GST-MITF

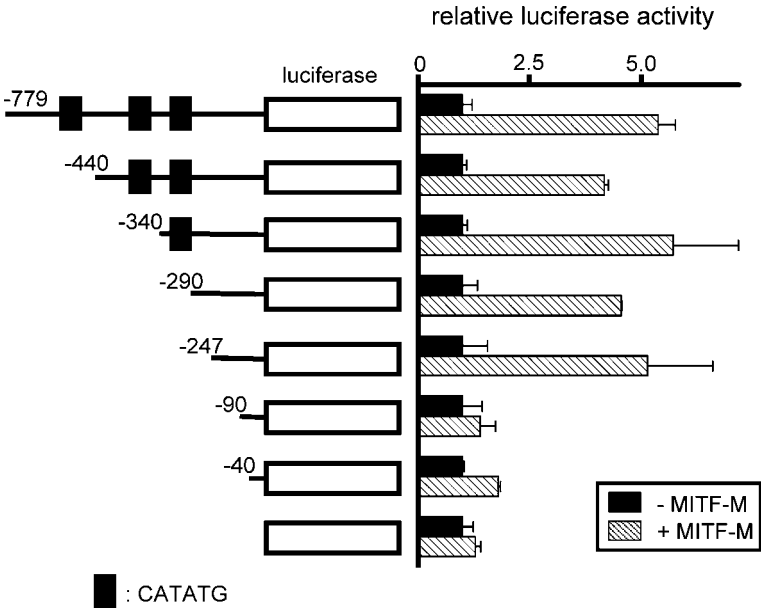

Fig. 1. Transcriptional activation of mMCP-9 promoter containing no CANNTG motif by MITF-M. The luciferase gene under the control of sequentially deleted mMCP-9 promoter was transiently transfected in HepG2 cells. Cells were also co-transfected with MITF-M and β-galactosidase. Data are means ± SD of triplicates from a representative experiment.

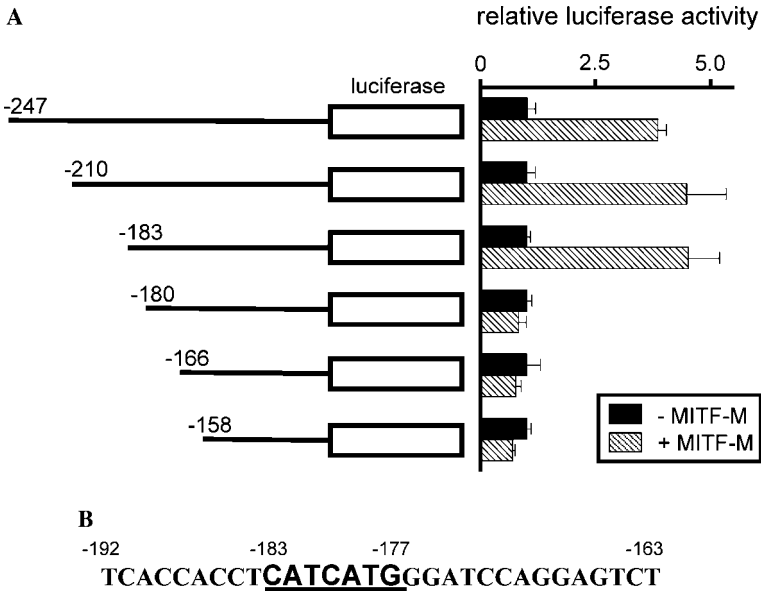

Fig. 2. Crucial region of the mMCP-9 promoter for MITF-M-induced transcription. (A) The luciferase gene under the control of sequentially deleted mMCP-9 promoter was transiently transfected in HepG2 cells. Cells were also co-transfected with MITF-M and β-galactosidase. Data are means ± SD of triplicates from a representative experiment. (B) The nucleotide sequence of the mMCP-9 promoter nt −192 to −163. The CANNNTG sequence is underlined.

but not GST produced two bands, which were competed away by excess of the unlabeled DNA fragment (Fig. 4A). These may represent different conformations or oligomeric states of the proteins, or they may result from proteolysis during EMSA. In contrast, formation of the protein–DNA complex was not competed by pre-incubation with the unlabeled DNA fragment containing mutations of TG to CT (Fig. 4A, lanes 5 and 6). To examine direct binding of MITF with the CATC ATG sequence, oligonucleotide probe consisting of quadruple of CATCATG was used. GST-MITF also formed a complex with the probe, which was inhibited by pre-incubation with the unlabeled oligonucleotide (Fig. 4B). The unlabeled fragment with mutations of TG to CT at 4- and 20-fold excess of the probe could not compete away complex formation between MITF and

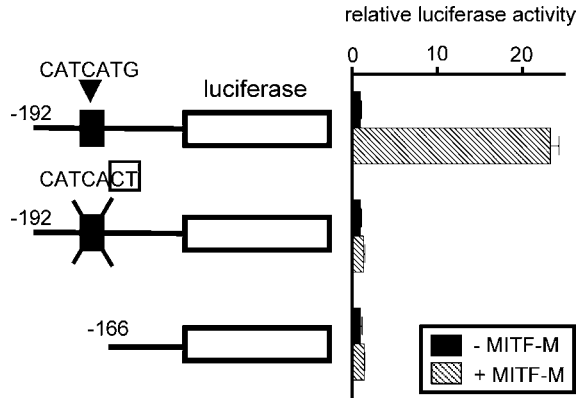

Fig. 3. Role of the CATCATG sequence located at nt −183 to −177 for MITF-M-induced transcription. The luciferase gene under the control of deleted or mutated mMCP-9 promoter was transiently transfected in HepG2 cells. Cells were also co-transfected with MITF-M and β-galactosidase. Data are means ± SD of triplicates from a representative experiment.

the labeled probe, although it did partially compete at a 75-fold excess of the probe (Fig. 4B).

Expression of MITF isoforms in mast cells and the activities of transactivation

The MITF encoded by the mutant *mi* allele (*mi*-MITF) deletes one of the four consecutive arginines in the basic domain and acts as a dominant-negative mutant [2,18,19]. The *mi/mi* mutant mice show microphthalmia, depletion of pigment in both hair and eyes, osteopetrosis, and decrease in the number of mast cells [5,20]. To examine gene expression of mMCP-9 in CMC from wild-type C57BL/6 mice and from *mi/mi* mutant mice, RT-PCR was performed. As shown in Fig. 5A, gene transcripts of mMCP-9 were not detected in CMC from *mi/mi* mice, in contrast to the clear expression in CMC from wild-type C57BL/6 mice. When a cDNA construct bearing *mi*-MITF was co-transfected with mMCP-9(-192)-lux, luciferase expression was not increased (Fig. 5C), suggesting that the insignificant gene expression of mMCP-9 in CMC from *mi/mi* mice resulted from failure of *mi*-MITF for the transcriptional activation of mMCP-9.

Recent studies revealed that MITF has several isoforms [2–4,19,21–23]. To examine expression of gene transcripts of MITF isoforms in CMC and melanoma cells, RT-PCR analyses were performed. As shown in Fig. 5B, CMC from wild-type C57BL/6 mice expressed all MITF isoforms examined, i.e., MITF-M, -mc, -E, and -H. RT-PCR analyses using CMC from the *mi/mi* mutant mice also revealed similar results to those using CMC from wild-type C57BL/6 mice. In contrast, B16 cells, a melanoma cell line, expressed only MITF-M and -E, although a faint band for MITF-H was detected (Fig. 5B).

Transcriptional activation of mMCP-9 by MITF isoforms was examined. Similar to MITF-M, overexpression of MITF-E resulted in a 59-fold increase in luciferase expression, when mMCP-9(-192)-lux was used as a reporter gene. An increase in luciferase activity was also detected by overexpression of MITF-mc, although the stimulatory activity was weaker (5-fold increase). In contrast, when the reporter construct containing point mutations of the CATCATG sequence was used, luciferase expression did not increase in response to overexpression of any of the MITF isoforms (Fig. 5C). Similar results were obtained also by overexpression of TFE3, another member of the bHLHLZ family.

Transcriptional activation of mMCP-9 was also examined in MC/9 mast cells, which express endogenous mMCP-9 gene (data not shown). When mMCP-9(-192)-

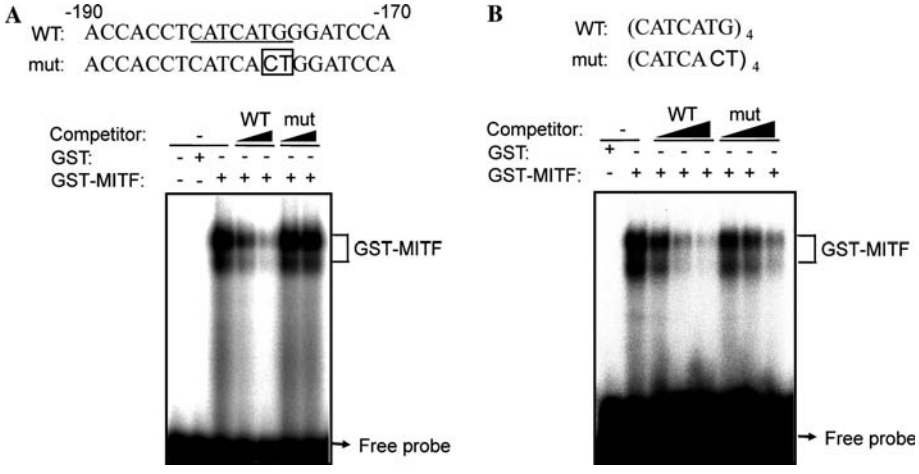

Fig. 4. Binding of MITF-M to the CATCATG sequence. (A) Nucleotide sequence spanning nt −190 to −170 of mMCP-9 was used as ^{32}P-labeled probe. Purified protein was pre-incubated with 4- or 20-fold excess of unlabeled oligonucleotide, followed by incubation with the probe. (B) A quadruple of the CATCATG sequence was used as ^{32}P-labeled probe. Purified protein was pre-incubated with 4-, 20- or 75-fold excess of unlabeled oligonucleotide, followed by incubation with the probe.

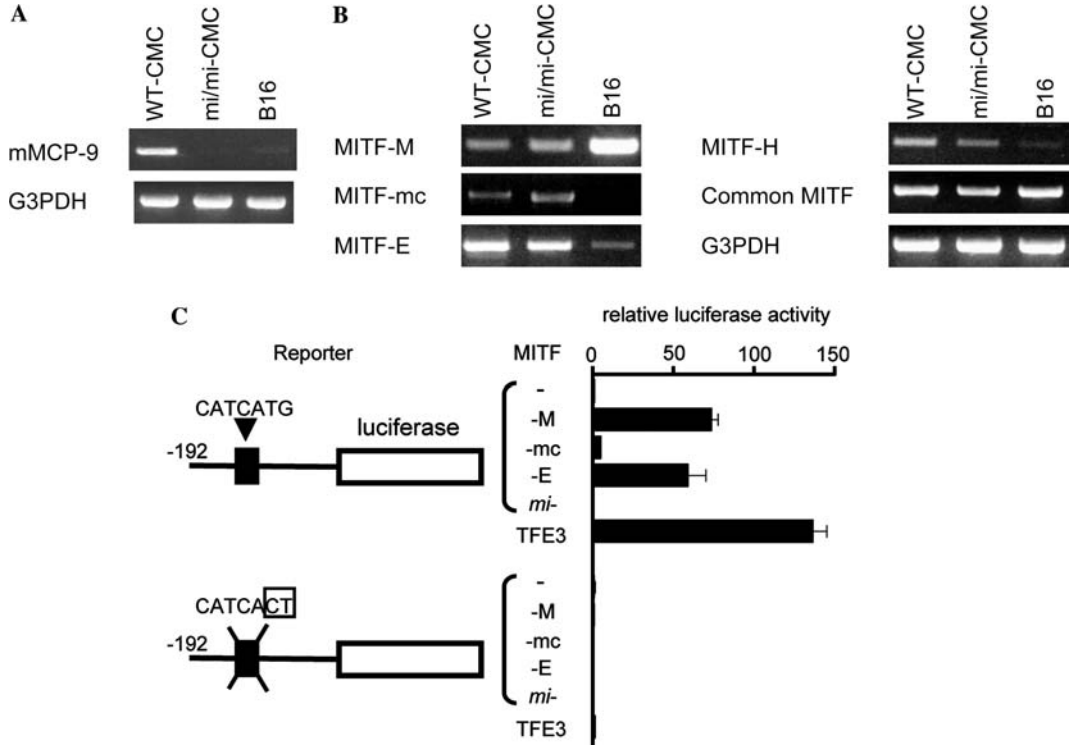

Fig. 5. Expression of mMCP-9 and MITF isoform in mast cells and transcriptional control of mMCP-9 by MITF isoforms. Gene expression of mMCP-9 (A) and MITF isoform (B) in CMC from wild-type C57BL/6 mice or *mi/mi* mutant mice and in B16 melanoma cells was examined by RT-PCR. (C) The reporter construct containing the mMCP-9 promoter starting from −192 or the mutated reporter construct was transiently transfected in HepG2 cells. Cells were also co-transfected with β-galactosidase and MITF isoform or TFE3. Data are means ± SD of triplicates from a representative experiment.

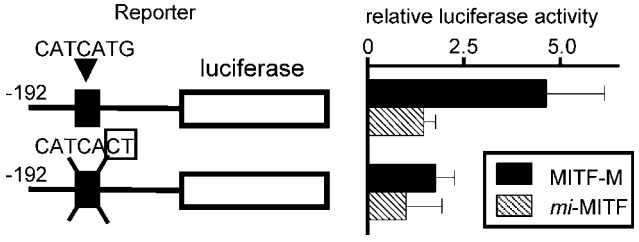

Fig. 6. Role of the CATCATG sequence located at nt −183 to −177 for MITF-induced transcription. The reporter construct containing the mMCP-9 promoter starting from −192 or the mutated reporter construct was transiently transfected in MC/9 mast cells. Cells were also co-transfected with β-galactosidase. Data are means ± SD of triplicates from a representative experiment.

lux was used as a reporter gene, overexpression of MITF-M but not *mi*-MITF resulted in the increase in luciferase expression. In contrast, MITF-M did not activate transcription of the reporter containing point mutations of the CATCATG sequence (Fig. 6).

Discussion

Here, we demonstrate that: (1) mMCP-9 gene transcription is positively regulated by MITF, (2) the most significant segment for the transcriptional activation is a nucleotide sequence, CATCATG, located between nt −184 and −176 of 5′-flanking region of the mMCP-9 gene, and (3) MITF directly binds to this segment. MITF is a member of the bHLHLZ family [2] and transcription factor members of this family bind to the E-box, a CANNTG sequence, to regulate gene transcription [5,17]. The present results indicate that MITF binds also to a nucleotide sequence similar to the CANNTG motif in which an extra base has been inserted and activates gene transcription in mast cells. Although Aksan and Goding [24] showed that MITF-M preferentially bound to the M-box, a AGTCATGTGCT sequence, which includes the E-box, the crucial region for the transcriptional activation of mMCP-9 gene does include neither the M-box nor the M-box-like sequence. This transcriptional regulation of mMCP-9 gene is distinct from those of mMCP-4 [6], -5 [7], and -6 [8], which was positively regulated through the CANNTG sequence through the binding of MITF. The novel recognition sites bound by MITF imply the importance of nucleotide sequences other than the E-box for transcriptional regulation by the bHLHLZ family. In fact, another member of the family, TFE3, also induced gene expression of mMCP-9 and the critical region for the transcription was the same as that for MITF.

Mouse MITF gene contains at least five isoform-specific first exons and exons 2–9 of all examined MITF are identical [3,22,23]. Therefore, the amino terminal amino acid sequence is different among MITF isoforms. While MITF-M, -mc, and -E activated transcription of mMCP-6 gene, MITF-M but not MITF-mc activated transcription of tyrosinase [3,4], suggesting that MITF isoforms differentially regulate gene transcription. In the present study, all examined MITF isoforms could activate transcription of mMCP-9. These results suggest that the amino terminus of MITF isoforms does not play a crucial role in the transcriptional activation of mMCP-9 and -6 genes, whereas it is important for tyrosinase transcription.

On the basis of amino acid sequence, mMCP-9 is predicted to be a chymase [25]. However, neither the enzymatic character nor the biological role is fully understood. In the helminth-infected mice, mMCP-9-positive mast cells were transiently detected in submucosa and in the crypt lamina propria of the jejunum during the inductive phase of inflammation [25,26]. In non-infected mice, mMCP-9 is localized in uterine mast cells [25]. Uterine mast cells and their mediators potentially play a role in the development of the uterus [27]. It is likely that mMCP-9 is involved in the onset of inflammation and in the tissue remodeling of the uterus during pregnancy. In fact, CMC from *mi/mi* mutant mice did not express mMCP-9 and *mi*-MITF failed to activate transcription of mMCP-9 in this study. Furthermore, in a previous study, it was shown that inversion of the uterus commonly occurs during deliveries in *mi/mi* mutant mice [28].

Recently, Ge et al. [29] reported transcriptional control of mMCP-9 by MITF. In the results of luciferase-based reporter assays using a series of deletion constructs and a mutated construct, the most significant finding was the CATATG sequence spanning nt −381 to −376 of mMCP-9 promoter. This result was inconsistent with our results. The reason for the discordant results is unknown, although the lower luciferase induction in their study (∼2-fold) [29] may be caused by inconsistent results.

Acknowledgments

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