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# Independent Influence of Strain Difference and *mi* Transcription Factor on the Expression of Mouse Mast Cell Chymases

Yi Ge,* Tomoko Jippo,* Young-Mi Lee,*†
Shiro Adachi,* and Yukihiko Kitamura*

*From the Department of Pathology,* Osaka University Medical School, Suita, Japan; and the Department of Oriental Pharmacy,† College of Pharmacy, Wonkwang University, Chonbuk, Republic of Korea*

**Expression of mouse mast cell protease (mMCP) genes was examined with particular attention to the transactivation effect of *mi* transcription factor (MITF) and the expression differences between C57BL/6 (B6) and WB strains. We had reported the enhancing effect of MITF on the expression of mMCP-4, -5, and -6 genes in cultured mast cells (CMCs) of B6 strain, and in the present study we demonstrated the enhancing effect on the expression of mMCP-2 and -9 genes as well. The enhancing effect of MITF on the expression of mMCP-2, -4, -5, -6, and -9 genes was also detected in CMCs of the WB strain. The regulation of mMCP-2, -4, and -9 genes was localized to a specific promoter element (CANNTG) which was recognized and bound by MITF and which was conserved between the B6 and WB strains. On the other hand, the expression of mMCP-2, -4, and -9 genes was smaller in CMCs of the B6 strain when compared to their expression in CMCs of the WB strain. Although mMCP-5 is a chymase as mMCP-2, -4, and -9, and genes encoding all of the chymases are located on chromosome 14, the mMCP-5 gene was regulated in a manner distinct from mMCP-2, -4, and -9 genes. *(Am J Pathol 2001, 158:281–292)***

Mast cells of mice contain various proteases. The complementary (c)DNAs and genes that encode mast cell carboxypeptidase A and nine of the 10 mouse mast cell-specific serine proteases have been cloned and sequenced.[1–11] The mast cell carboxypeptidase A is an exopeptidase that prefers C-terminal aliphatic amino acids. The mouse mast cell proteases (mMCPs) -1, -2, -4, -5, and -9 are predicted to be chymases from the deduced amino acid sequences, whereas mMCP-6, -7, and transmembrane tryptase to be tryptases. The mMCP-1, -2, -4, -5, and -9 genes reside on chromosome 14 and link with a gene complex encoding blood cell proteases, such as the cathepsin G and multiple granzymes.[11–15] On the other hand, the mast cell carboxypeptidase A gene resides on the chromosome 3, and the mMCP-6, -7, and transmembrane tryptase genes reside on the chromosome 17.[12] Chromosomal localization of mMCP-8 has not been reported. From the viewpoint of structure, mMCP-8 is more closely related to cathepsin G and granzymes than to mast cell chymases.[10]

The protease expression phenotype is influenced not only by extracellular environmental factors[16–19] but also by intracellular factors.[20–22] As extracellular factors, effects of tissue environments and cytokines have been studied. Messenger RNA (mRNA) of mMCP-2 is expressed by mast cells in the mucosal layer of the stomach of (WB × C57BL6) $F_1$ (hereafter called WBB6$F_1$)-+/+ mice but not by mast cells in the muscle layer of the stomach.[16] Addition of interleukin 9, interleukin 10, or transforming growth factor-$\beta_1$ significantly increased the mRNA expression of both mMCP-1 and -2 in cultured mast cells (CMCs).[9,17,18] On the other hand, addition of stem cell factor increased the expression of mMCP-4 and -6 in CMCs.[19]

Transcription factors that are involved in the transactivation of mMCP genes are intracellular factors. The *mi* transcription factor (hereafter called MITF) is a member of the basic helix-loop-helix leucine zipper (bHLH-Zip) protein family and is encoded by the *mi* locus of mice.[23,24] The MITF encoded by the mutant *mi* locus deletes one of four consecutive arginines in the basic domain.[23,25,26] We showed that the mRNA expression of the mMCP-4, -5, and -6 was deficient in CMCs derived from C57BL/6 (B6)-*mi/mi* mice.[20–22]

Strain difference also affects the expression of mMCP genes. When considered as a whole mouse, the strain difference may be either an extracellular or intracellular factor. Amounts of secreted cytokines might be different among mouse strains. However, when protease expres-

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Address reprint requests to Yukihiko Kitamura, M.D., Department of Pathology, Osaka University Medical School, Yamada-oka, 2-2, Suita 565-0871, Japan. E-mail: kitamura@patho.med.osaka-u.ac.jp.

sion phenotypes are compared among CMCs in the same culture condition, the strain difference may be an intracellular factor. Therefore, both the strain differences and transcription factors are considered to be intracellular factors that may affect the protease expression phenotype of CMCs.

We have studied the effect of MITF only in the B6 strain.[20–22] The differences between B6-+/+ mice and B6-*mi/mi* mice have been examined. On the other hand, the expression of mMCP-2 and -4 genes was much higher in CMCs derived from WB-+/+ mice than in CMCs of B6-+/+ mice.[16,27] The effect of MITF on the expression of mMCP-2 and -4 genes remained to be examined in the WB strain. In the present study, we crossed WB-+/+ mice to B6-*mi*/+ mice to obtain the mice that possessed the WB strain-derived mMCP-2 genes and the double gene dose of mutant *mi* allele. The expression of the WB strain-derived mMCP-2 gene was significantly lower in mice of *mi/mi* genotype than in mice of +/+ genotype. We also examined the expression of WB strain-derived mMCP-4 and -9 genes in mice of *mi/mi* genotype.

## Materials and Methods

### Mice

WB-+/+ and B6-+/+ mice were raised in our laboratory. The original stock of B6-*mi*/+ mice was purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in our laboratory by consecutive back-crosses to our own inbred B6 colony (more than 19 generations at the time of the present experiment). We selected female and male B6-*mi*/+ mice by the presence of a white belly spot, and crossed together. The resulting B6-*mi/mi* mice were selected by their white coat color.[28,29]

The alleles of mMCP-2, -4, and -9 genes possessed by the B6 strain were described as $P2^{B6}$, $P4^{B6}$, and $P9^{B6}$. And the alleles of mMCP-2, -4, and -9 genes of the WB strain were described as $P2^{WB}$, $P4^{WB}$, and $P9^{WB}$. In the present experiment, mice of ($P2^{WB}/P2^{WB}$, $P4^{WB}/P4^{WB}$, $P9^{WB}/P9^{WB}$, *mi/mi*) and control ($P2^{WB}/P2^{WB}$, $P4^{WB}/P4^{WB}$, $P9^{WB}/P9^{WB}$, +/+) genotype were necessary. First, we crossed WB-($P2^{WB}/P2^{WB}$, $P4^{WB}/P4^{WB}$, $P9^{WB}/P9^{WB}$, +/+) mice with B6-($P2^{B6}/P2^{B6}$, $P4^{B6}/P4^{B6}$, $P9^{B6}/P9^{B6}$, *mi*/+) mice. Then, we selected WBB6$F_1$- ($P2^{WB}/P2^{B6}$, $P4^{WB}/P^{B6}$, $P9^{WB}/P9^{B6}$, *mi*/+) mice by the presence of a white belly spot. Then we back-crossed the $F_1$ hybrid mice to WB-($P2^{WB}/P2^{WB}$, $P4^{WB}/P4^{WB}$, $P9^{WB}/P9^{WB}$, +/+) mice and obtained N2 mice. We selected N2 mice possessing the mutant *mi* allele (ie, N2-*mi*/+) by the presence of a remarkable white belly spot. We crossed together these N2-*mi*/+ mice, and finally selected mice of ($P2^{B6}/P2^{B6}$, $P4^{B6}/P4^{B6}$, $P9^{B6}/P9^{B6}$, *mi/mi*) (all white), ($P2^{B6}/P2^{B6}$, $P4^{B6}/P4^{B6}$, $P9^{B6}/P9^{B6}$, +/+) (all black), ($P2^{WB}/P2^{WB}$, $P4^{WB}/P4^{WB}$, $P9^{WB}/P9^{WB}$, *mi/mi*) (all white), and ($P2^{WB}/P2^{WB}$, $P4^{WB}/P4^{WB}$, $P9^{WB}/P9^{WB}$, +/+) (all black) by the coat color and the sequences of genomic DNAs of mMCP-2, -4, and -9.

### Sequence of Genomic DNAs

Genomic DNAs were obtained from tail tip with the conventional phenol extraction method. Each genomic DNA was amplified in 25 $\mu$l of polymerase chain reaction (PCR) mixture containing 0.125 U of *Taq* DNA polymerase (Takara Shuzou, Kyoto, Japan) and 12.5 pmol each of sense (5′-GCCTATCTGAAGTTCACCAC-3′, nucleotides +628 to +647, +1 is the transcription initiation site) and antisense (5′-ATGGCAAGTTGGCACTCTAC-3′, nucleotides +988 to +1007)[12] primers for the mMCP-2 gene, sense (GGAGACTCTGGAGGACCTCT-3′, nucleotides +2209 to +2228) and antisense (5′-ACAGGGAACAGTCCATCATC-3′, nucleotides +2526 to +2545)[4] primers for the mMCP-4 gene, and sense (5′-GCTAACTTGACTTCTGCTGTGG-3′, nucleotides +1617 to +1638) and antisense (5′-GGGTTATTAGAAGAGCTCTGGC-3′, nucleotides +2445 to +2466)[11] primers for the mMCP-9 gene by 28 cycles of denaturation at 94°C (30 seconds), annealing at 55°C (30 seconds), and synthesis at 72°C (1 minute). Three nucleotides in this portion (nucleotides +628 to +728) of mMCP-2 cDNA were different between WB and B6 strains. Six nucleotides in this portion (nucleotides +2209 to +2545) of mMCP-4 cDNA were different between WB and B6 strains, and moreover the other 11 nucleotides were deleted in B6 strain. Three nucleotides in this portion (nucleotides +1617 to +2466) of mMCP-9 genomic DNA were different between WB and B6 strains. The PCR products were subcloned into the Bluescript KS (−) plasmids (pBS; Stratagene, La Jolla, CA) for further analysis. Nucleotide sequence was determined as described previously.[30] Strain-dependent differences of cDNA sequences were compared using the DNASIS sequence analysis program (Hitachi Software Engineering Co., Ltd., Tokyo, Japan).

### Cells

Pokeweed mitogen-stimulated spleen cell-conditioned medium (PWM-SCM) was prepared according to the method described by Nakahata et al.[31] Mice 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 $\alpha$-minimal essential medium ($\alpha$-MEM; ICN Biochemicals, 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 7 days. More than 95% of cells were CMCs 4 weeks after the initiation of the culture.[32,33] The helper virus-free packaging cell line ($\psi$2) was maintained in Dulbecco's modified Eagle's medium (DMEM, ICN Biomedicals) supplemented with 10% FCS.[34] The IC-2 cell line was provided by Dr. I. Yahara (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan)[35] and maintained in $\alpha$-MEM supplemented with 10% PWM-SCM and 10% FCS.

### Sequence of mMCP-2, -4, and -9 cDNAs

Five micrograms of total RNA obtained from WB-+/+ CMCs or B6-+/+ CMCs were reverse-transcribed in 20

μl of the reaction mixture containing 20 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) and random hexamer. One μl of each reaction product was amplified in 25 μl of PCR mixture containing 0.125 U of *Taq* DNA polymerase and 12.5 pmol each of sense (5′-AGAAGCTCACCAAGGCCTCA-3′, nucleotides +1 to +20) and antisense (5′-GCAAGAGGTTAGGTCTTTATTG-3′, nucleotides +2490 to +2511)[12] primers for the mMCP-2 gene, sense (5′-AGAATCTCTCTCCAAGCTGTGACCG-3′, nucleotides +1 to +25) and antisense (5′-GGAGGTTAGGTCTTTACTGAGGTGCA-3′, nucleotides +2548 to +2573)[4,36] primers for the mMCP-4 gene, and sense (5′-AAAATCTCTCTCCAGACTGCGA-3′, nucleotides +1 to +22) and antisense (5′-AAGACTCTGATGCACGCAGGTCA-3′, nucleotides +2219 to +2241)[11] primers for the mMCP-9 gene by 30 cycles of denaturation at 94°C (30 seconds), annealing at 57°C (30 seconds), and synthesis at 72°C (1 minute). Inserts were subcloned into the plasmid pBS for further analysis. Nucleotide sequence was determined as described previously.[30]

## *Northern Blot Analysis*

Thirty micrograms of total RNA from CMCs were prepared by the lithium chloride-urea method.[37] Northern blot analysis was performed using mMCP-2,[3,12] -4,[4] -5,[5] -6,[6] -9,[11] and β-actin[38] cDNAs labeled with α-[$^{32}$P]dCTP (DuPont/NEN Research Products, Boston, MA) by random oligonucleotide priming as probes. After hybridization at 42°C, blots were washed to a final stringency of 0.2× standard saline citrate (1× standard saline citrate is 150 mmol/L NaCl and 15 mmol/L trisodium citrate, pH 7.4) at 50°C and subjected to autoradiography.

## *Construction of Retrovirus Vector and Its Infection*

Plasmid pBS containing the whole coding region of +-MITF or *mi*-MITF (pBS-+-MITF and pBS-*mi*-MITF, respectively) had been constructed in our laboratory.[39,40] A retroviral vector, pM5Gneo,[41] a derivative of myeloproliferative sarcoma virus vector, was a kind gift from Dr. W. Ostertag (Universität Hamburg, Hamburg, Germany). The purified *Sma*I-*Hin*cII fragment from pBS-+-MITF or pBS-*mi*-MITF was introduced into the blunted *Eco*RI site of pM5Gneo. The resulting pM5Gneo-+-MITF and pM5Gneo-*mi*-MITF were transfected into the packaging cell line (ψ*2*)[34] by the calcium phosphate precipitation method,[42] and neomycin-resistant ψ*2* cell clones were selected by culturing in DMEM supplemented with 10% FCS and G418 (0.8 mg/ml; Life Technologies, Inc., Grand Island, NY). For gene transfer, spleen cells obtained from *mi/mi* mice were incubated on an irradiated (30 Gy) subconfluent monolayer of virus-producing ψ*2* cells for 72 hours in α-MEM supplemented with 10% PWM-SCM and 10% FCS. Neomycin resistant CMCs were obtained by continuing the culture in α-MEM containing 10% PWM-SCM, 10% FCS, and G418 (0.8 mg/ml) for 4 weeks.

## *Construction of Reporter Plasmids*

The luciferase gene subcloned into pSP72 (pSPLuc)[43] was generously provided by Dr. K. Nakajima (Osaka City University Medical School, Osaka, Japan). To construct reporter plasmids, a DNA fragment containing a promoter region and the first exon (noncoding region) of the mMCP-2 gene (nucleotides −1311 to +21)[12] and mMCP-9 gene (nucleotides −1191 to +10)[11] obtained from genomic DNA of B6-+/+ mouse were cloned into the upstream region of the luciferase gene in pSPLuc. The deletion of the mMCP-2 or -9 promoter was done by using the appropriate restriction enzyme. The mutations were introduced by PCR with mismatched primers. Deleted or mutated products were verified by sequencing.

## *Transient Assay*

The expression vector containing the β-galactosidase gene was used as an internal control. Because IC-2 cells expressed effector gene by themselves,[22] the reporter and the expression vector containing the β-galactosidase gene were added to cell suspension ($1 \times 10^7$) in 0.7 ml phosphate-buffered saline, mixed gently, and incubated on ice for 10 minutes. For gene transfer, cells were electroporated by a single pulse (975 microfarads at 350 V) from a Gene Pulser II (Bio-Rad Laboratories, Richmond, CA). After incubation on ice for 10 minutes, the cells were suspended in 10 ml of complete culture medium. IC-2 cells were harvested 8 hours after transfection. Cells were lysed with 0.1 mol/L potassium phosphate buffer (pH 7.4) containing 1% Triton X-100 (Sigma, St Louis, MO). Extracts were then used to assay luciferase activity with luminometer model LB96P (Berthold, Wildbad, Germany) and β-galactosidase activity. Luciferase activity was normalized by β-galactosidase activity and total protein concentration according to the method described by Yasumoto et al.[44]

## *Electrophoretic Gel Mobility Shift Assay (EGMSA)*

The production and purification of glutathione-*S*-transferase (GST) -+-MITF and GST-*mi*-MITF fusion proteins were described previously.[39,40] Oligonucleotides were labeled with α-[$^{32}$P]dCTP by filling 5′-overhangs, and were used as probes for EGMSA. DNA binding assays were performed in a 20 μl reaction mixture containing 10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L ethylenediaminetetraacetic acid, 75 mmol/L KCl, 1 mmol/L dithiothreitol, 4% Ficoll type 400, 50 ng poly (dI-dC), 25 ng of the labeled DNA probe, and 3.5 μg of GST-+-MITF or GST-*mi*-MITF fusion protein. After 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× TBE buffer (1× TBE is 90 mmol/L Tris-HCl, 64.6 mmol/L boric acid, and 2.5 mmol/L ethylenediaminetetraacetic acid, pH 8.3). The polyacrylamide gels were dried on Whatman 3MM chromatography paper (Whatman, Maidstone, UK) and subjected to autoradiography.

Competitive DNA binding assays were performed as described above, except that the unlabeled competitive DNA was added to the reaction mixture before addition of GST-+-MITF fusion protein.

### *Semiquantitative Reverse Transcriptase (RT) Modification of PCR*

Various amounts of total RNA (5.0, 0.5, and 0.05 μg) obtained from CMCs derived from WB-+/+ or B6-+/+ mice were reverse-transcribed in 20 μl of the reaction mixture containing 20 U of avian myeloblastosis virus reverse transcriptase and random hexamer. One μl of each reaction product was amplified in 25 μl of PCR mixture containing 0.125 U of *Taq* DNA polymerase and 12.5 pmol each of sense (5′-CTGATCTGGTGAATCGGATC-3′, nucleotides +1051 to +1070)[23] and antisense (5′-TCCTGAAGAAGAGAGGGAGC-3′, nucleotides +1422 to +1441)[23] primers for MITF gene by 28 cycles of 30 seconds of denaturation at 94°C, 30 seconds of annealing at 55°C, and 1 minute of synthesis at 72°C. Ten μl of the PCR products was electrophoresed in 1.0% agarose gel containing ethidium bromide.

## *Results*

### *Effect of MITF on the Expression of mMCP-2 and -9 Genes in B6 Mice*

#### *Low Expression of mMCP-2 and -9 Genes in B6-mi/mi Mice*

We already reported that the expression of the mMCP-4, -5, and -6 was significantly higher in CMCs derived from B6-+/+ mice than those derived from B6-*mi/mi* mice.[20–22] In the present experiment, the mRNA expression of mMCP-2 and -9 genes was compared between B6-+/+ CMCs and B6-*mi/mi* CMCs. The mRNA expression of mMCP-2 and -9 was significantly higher in B6-+/+ CMCs than in B6-*mi/mi* CMCs (Figure 1).

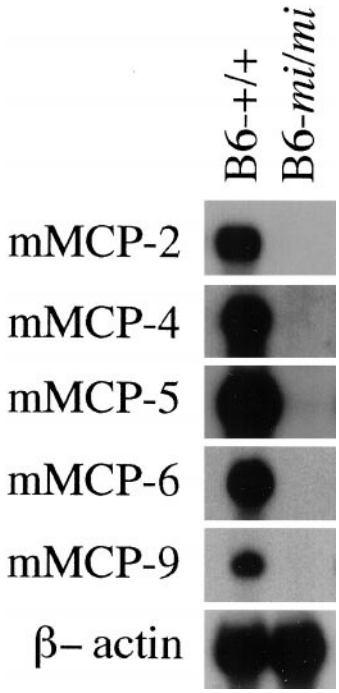


**Figure 1.** Expression of mMCP-2, -4, -5, -6, and -9 mRNA transcripts in CMCs of B6-+/+ and B6-*mi/mi* mice. Total RNAs were extracted from CMCs of B6-+/+ and B6-*mi/mi* mice, and 20 μg of total RNA was electrophoresed and hybridized with each probe. Three similar experiments were done, and a representative result is shown. Comparable results were obtained in two other experiments.

#### *Effect of Introduction of cDNA Encoding +-MITF or mi-MITF*

To examine the involvement of +-MITF in the expression of the mMCP-2 and -9 genes, we introduced cDNA encoding +-MITF or *mi*-MITF to CMCs of B6-*mi/mi* mouse origin. Overexpression of either +-MITF or *mi*-MITF was confirmed in the B6-*mi/mi* CMCs. The poor mRNA expression of the mMCP-2 and -9 genes was normalized in the B6-*mi/mi* CMCs overexpressing +-MITF but not in the B6-*mi/mi* CMCs overexpressing *mi*-MITF (Figure 2).

#### *Transactivation Effect of +-MITF on the mMCP-2 Promoter*

MITF is a member of bHLH-Zip protein family transcription factors and may recognize and bind various CANNTG motifs (any nucleotides are possible at position N).[45] The mMCP-2 promoter starting from nucleotides −1311 have three CANNTG motifs, CAAGTG (nucleotides −1304 to −1299), CACATG (nucleotides −377 to −372), and CATATG (nucleotides −316 to −311). We introduced the luciferase gene under the control of various mMCP-2 promoters into the IC-2 mast cell line that expressed +-MITF.[22] Luciferase activity was enhanced after introduction of the reporter plasmid starting from either nucleotide −1311 or nucleotide −379 (Figure 3). Then, the deleted reporter plasmid containing the mMCP-2 promoter starting from nucleotide −364 or nucleotide −310 was constructed and introduced into IC-2 cells. In either case, the luciferase activity was not enhanced (Figure 3). We mutated either the CACATG motif (nucleotides −377 to −372) to CTCAAG or the CATATG (nucleotides −316 to −311) motif to CTTAAG in the reporter plasmid starting from nucleotide −379. The luciferase activity was not enhanced when the CACATG motif (nucleotides −377 to −372) was mutated (Figure 3). On the other hand, the luciferase activity remained to be enhanced when the CATATG motif (nucleotides −316 to −311) was mutated (Figure 3). The luciferase activity was not enhanced either when both CACATG (nucleotides −377 to −372) and CATATG (nucleotides −316 to −311) motifs were mutated (Figure 3). Only the CACATG motif of the mMCP-2 promoter seemed to play a role for the enhancement of the mMCP-2 gene expression.

Next, the binding of GST-+-MITF to the oligonucleotides containing the CACATG motif (nucleotides −377 to −372) of the mMCP-2 promoter was examined by EGMSA. When the oligonucleotide containing the CA-


**Figure 2.** Reduced expression of mMCP-2 and -9 mRNAs in B6-*mi/mi* CMCs and normalization of the mMCP-2 and -9 expression in B6-*mi/mi* CMCs by the introduction of the +-MITF cDNA but not of *mi*-MITF cDNA. The blot was hybridized with $^{32}$P-labeled cDNA probe of MITF, mMCP-2, -9, or of $\beta$-actin. Three similar experiments were done, and a representative result is shown. Comparable results were obtained in two other experiments.

CATG motif (oligo 1) was used as a probe, the specific binding of GST-+-MITF was detected (Figure 4). The excess amount (200×) of the unlabeled oligonucleotide containing the CACATG motif (oligo 1) abolished the binding of the GST-+-MITF, whereas the excess amount (200×) of unlabeled oligonucleotide containing the mutated CTCAAG motif (oligo 2) did not inhibit the binding (Figure 4).

### *Transactivation Effect of +-MITF on the mMCP-9 Promoter*

We examined the interaction of +-MITF with 5′-upstream region of the mMCP-9 gene. The mMCP-9 promoter starting from nucleotide −1132 have five CANNTG motifs, CACATG (nucleotides −1099 to −1094), CATTTG

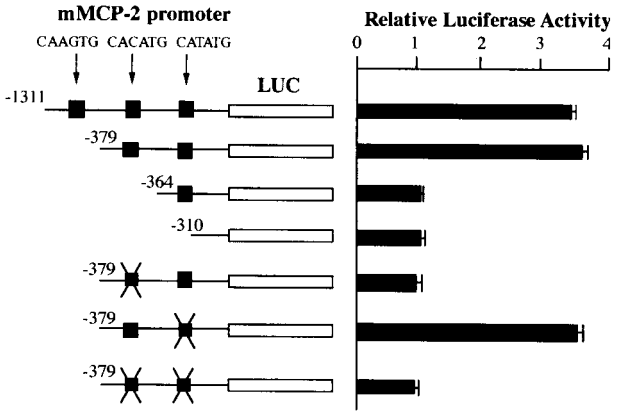


**Figure 3.** Luciferase reporter gene promoter assay in the IC-2 cell line which expressed +-MITF. The luciferase gene under control of the normal, deleted, or mutated mMCP-2 promoter was introduced into the IC-2 cells with electroporation. **Bars** indicate the SE of three assays.

(nucleotides −1054 to −1049), CAGCTG (nucleotides −782 to −777), CATATG (nucleotides −556 to −551) and CATATG (nucleotides −381 to −376). The reporter plasmid that contained the luciferase gene under the control of the mMCP-9 promoter starting from nucleotides −1132, −787, −390, −364 was constructed. We intro-

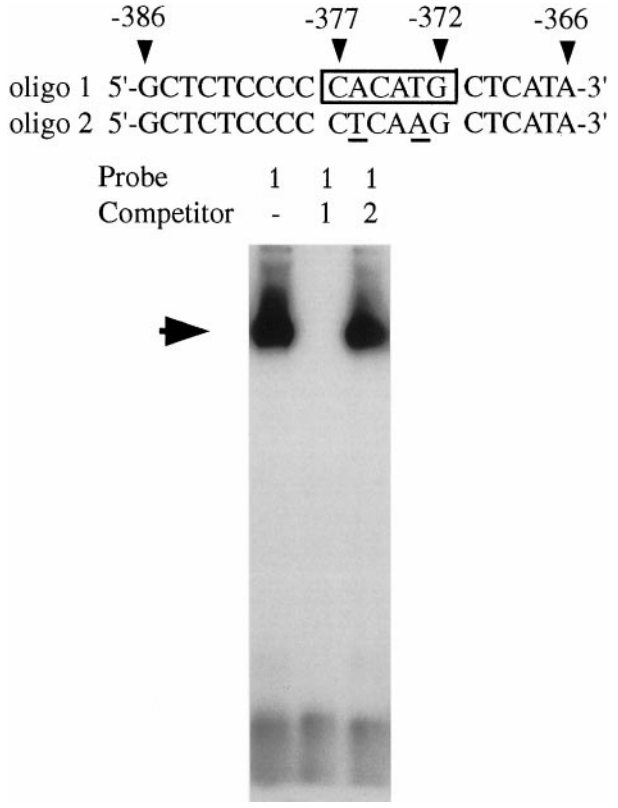


**Figure 4.** EGMSA using GST-+-MITF fusion protein. The labeled 5′-GCTCTCCCC*CACATG*CTCATA-3′ oligonucleotide (oligo 1, nucleotide −386 to −366) in the mMCP-2 promoter was used as a probe (CANNTG motif is **boxed**). The sequences of the mutated oligonucleotide is shown as oligo 2. The mutated nucleotides are **underlined**. The excess amount (200×) of unlabeled oligo 1 or oligo 2 was added as a competitor. The DNA-protein complexes are indicated by an **arrow**. Three similar experiments were done, and a representative result is shown. Comparable results were obtained in two other experiments.

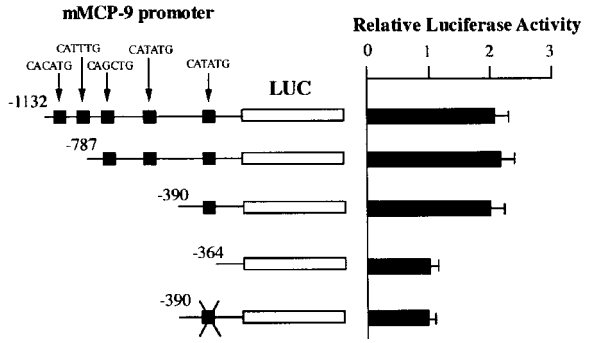


**Figure 5.** Luciferase reporter gene promoter assay in the IC-2 cell line that expressed +-MITF. The luciferase gene under control of the normal, deleted, or mutated mMCP-9 promoter was introduced into the IC-2 cells with electroporation. **Bars** indicate the SE of three assays.

duced the luciferase gene under the control of the mMCP-9 promoter into IC-2 cells. Luciferase activity was enhanced when we used the reporter plasmid containing the mMCP-9 promoter starting from nucleotides −1132, −787, and −390 (Figure 5). In contrast, the luciferase activity was not enhanced when we used the reporter plasmid starting from nucleotide −364. Transcription activity of the mMCP-9 promoter starting from nucleotide −390 was abolished when the CATATG motif (nucleotides −381 to −376) was mutated.

To examine whether the +-MITF protein practically bound the CATATG motif (nucleotides −381 to −376), EGMSA was performed with oligonucleotide containing the CATATG motif (oligo 1) as a probe. A retarded band was observed in the sample containing the oligo1 and GST-+-MITF (Figure 6). The excess amount (200×) of the unlabeled oligonucleotide containing the CATATG motif (oligo 1) abolished the binding of the GST-+-MITF, whereas the excess amount (200×) of unlabeled oligonucleotide containing the mutated CTTAAG motif (oligo 2) did not inhibit the binding (Figure 6).

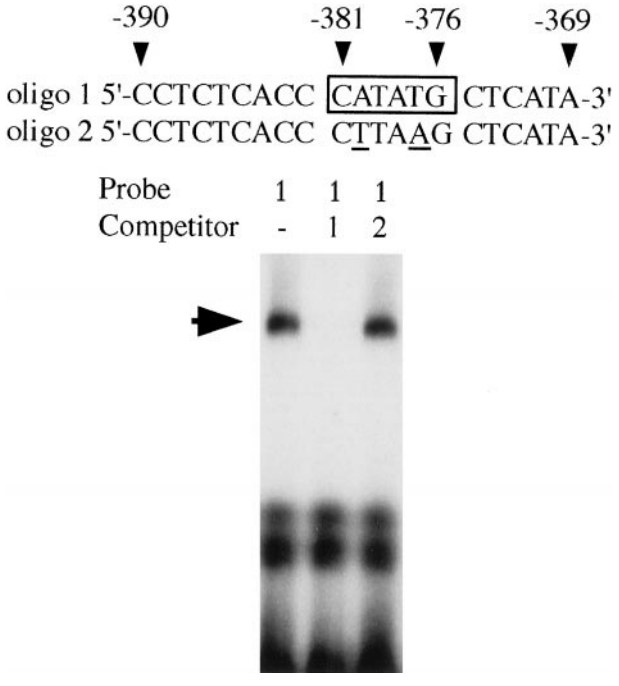


**Figure 6.** EGMSA using GST + MITF fusion protein. The labeled 5′-CCTCTCACC*CATATG*CTCATA-3′ oligonucleotide (oligo 1, nucleotide −390 to −369) in the mMCP-9 promoter was used as a probe (CANNTG motif is **boxed**). The sequences of the mutated oligonucleotide is shown as oligo 2. The mutated nucleotides are **underlined**. The excess amount (200×) of unlabeled oligo 1 or oligo 2 was added as a competitor. The DNA-protein complexes are indicated by an **arrow**. Three similar experiments were done, and a representative result is shown. Comparable results were obtained in two other experiments.

## *Effect of MITF on the Expression of the WB Strain-Derived mMCP-2, -4, and -9 Genes*

### *Expression of mMCP-2, -4, and -9 Genes in WB-+/+ Mice*

We have reported that the expression of mMCP-2 was much higher in CMCs derived from WB-+/+ mice than in CMCs derived from B6-+/+ mice, whereas the expression of mMCP-6 was comparable between CMCs derived from WB-+/+ mice and CMCs derived from B6-+/+ mice. As shown in Figure 7, the expression of mMCP-2, -4, and -9 genes was higher in CMCs of WB-+/+ mice than in CMCs of B6-+/+ mice, whereas the expression of mMCP-5 and -6 was comparable between WB-+/+ and B6-+/+ CMCs.

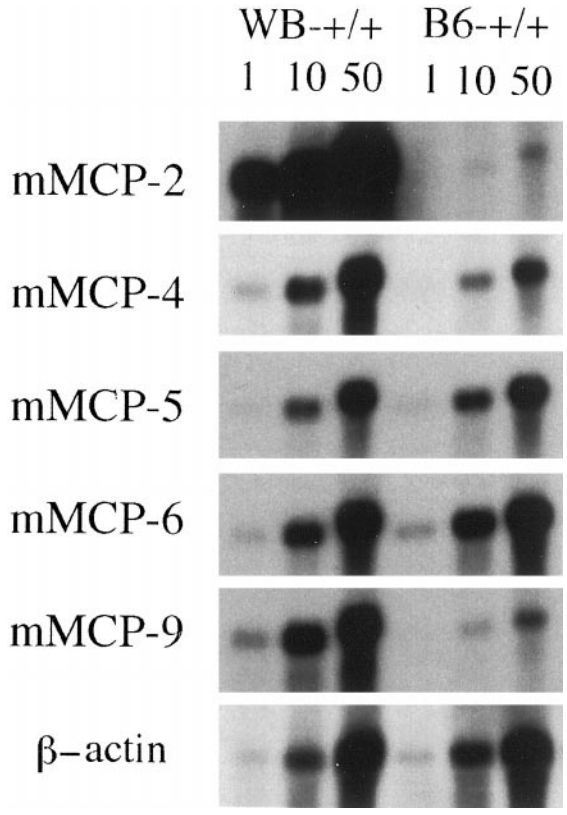


**Figure 7.** Expression of mMCP-2, -4, -5, -6, and -9 mRNA transcripts in CMCs of WB-+/+ and B6-+/+ mouse origin. Total RNA was extracted from CMCs of WB-+/+ and B6-+/+ mice, and 1, 10, and 50 μg of total RNA was electrophoresed and hybridized with each probe. Three similar experiments were done, and a representative result is shown. Comparable results were obtained in two other experiments.

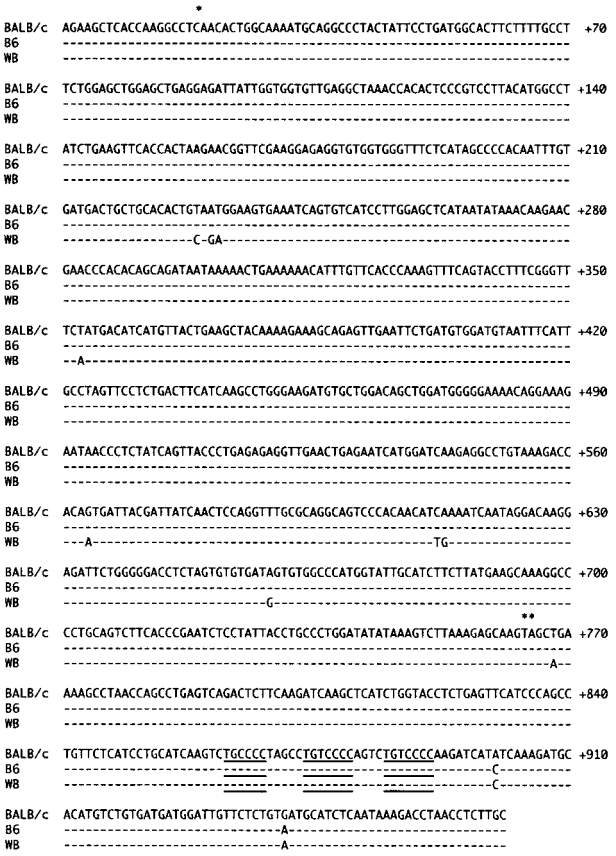


**Figure 8.** Comparison of the nucleotide sequence of mMCP-2 cDNA among BALB/c, B6, and WB strains. Numbering of the nucleotides begins at the transcription initiation site of the mMCP-2 cDNA of BALB/c strain. Each sequence was confirmed three times. **Dashes** indicate identical nucleotides. *, Translation-initiation site. **, Translation-termination site. The repetitive UGXCCCC sequence is **underlined**.

### *Strain Difference in Nucleotide Sequence of mMCP-2, -4, and -9 cDNAs*

The poor expression of mMCP-7 mRNA in the B6 strain was attributed to the presence of a stop codon.[46] Truncated transcript was unstable. The nucleotide sequences of mMCP-2, -4, and -9 cDNAs of the B6 strain was compared to those of the WB strain.

The complete nucleotide sequence of the mMCP-2 cDNA of BALB/c strain has been reported by Gurish et al.[12] We sequenced the mMCP-2 cDNA obtained from the CMCs of B6 strain and compared it to that of the BALB/c strain. Two nucleotides (nucleotides +900 and +941) were different (Figure 8), but those nucleotide changes did not result in the alternation of amino acids. Then we compared the nucleotide sequence of the mMCP-2 cDNA between BALB/c and WB strains. Eleven nucleotides (nucleotides +229, +231, +232, +353, +564, +612, +613, +659, +768, +900, and +941) were different, but four of 11 nucleotide changes did not result in the alternation of amino acids (Figure 8). The remaining seven nucleotide changes caused five amino acid alternations: AAT codon at nucleotides 230 to 232 changed to AGA, leading to an alternation from Asn to Arg; TAT codon at nucleotides 353 to 355 changed to AAT, leading to an alternation from Try to Asn; AGT codon at nucleotides 563 to 565 changed to AAT, leading to an alternation from Ser to Asn; TCA codon at nucleotides 611 to 613 changed to TTG, leading to an alternation from Ser to Leu; AGT codon at nucleotides 659 to 661 changed to GGT, leading to an alternation from Ser to Gly.

Serafin et al[4,36] have reported the sequence of mMCP-4 cDNA obtained from the KiSV-MC1 mastocytoma cell line, which was derived from DBA/2 strain. We sequenced the mMCP-4 cDNA obtained from CMCs of the B6 strain. When we compared the nucleotide sequence of mMCP-4 cDNA between DBA/2 and B6 strains, 11 nucleotides (nucleotides +217, +372, +515, +576, +624, +675, +762, +772, +828, +868, and +882) were different, but eight of 11 nucleotide changes did not result in the alternation of amino acids (Figure 9). The remaining three changes caused the amino acid changes; ATG codon at nucleotides 217 to 219 changed to TTG, leading to an alternation from Met to Leu; ACA codon at nucleotides 514 to 516 changed to ATA, leading to an alternation from Thr to Ile; GAG codon at nucleotides 772 to 774 changed to AAG, leading to an alterna-

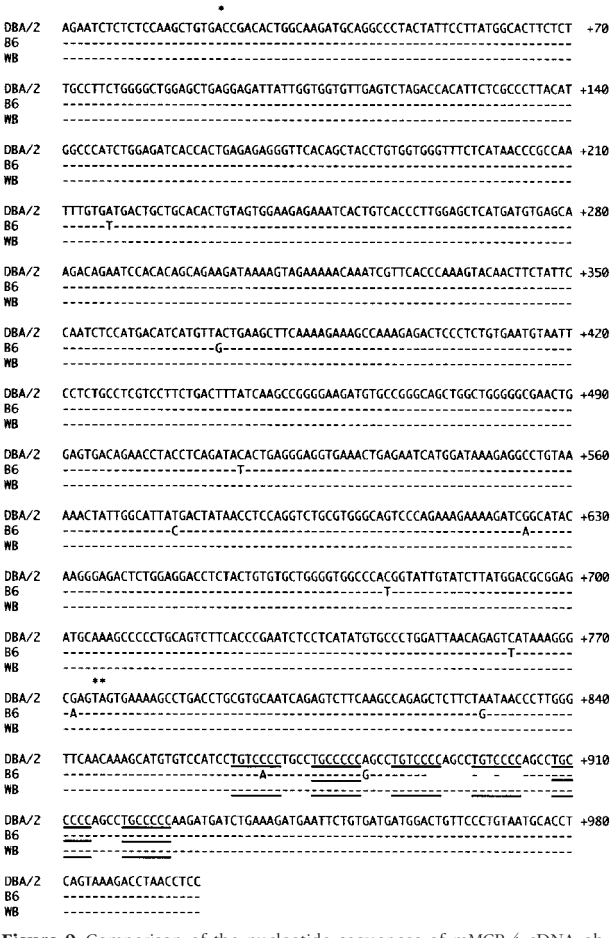


**Figure 9.** Comparison of the nucleotide sequences of mMCP-4 cDNA obtained from the KiSV-MC1 mastocytoma cell line of DBA/2 strain origin,[4,36] CMCs of B6 strain origin, and CMCs of WB strain origin. Numbering of the nucleotides begins at the transcription initiation site of the mMCP-4 cDNA of DBA/2 strain. Each sequence was confirmed three times. **Dashes** indicate identical nucleotides. **Blank** indicates the deletion in the sequence. *, Translation-initiation site. **, Translation-termination site. The repetitive UGXCCCC sequence is **underlined**.

tion from Glu to Lys. Moreover, another 11 nucleotides in the 3′ untranslated region (3′UTR) were deleted in B6 strain (nucleotides +891 to +896, nucleotides +898 to +899, nucleotides +901 to +903) (Figure 9). When we compared the nucleotide sequence between DBA/2 and WB strains, there were no differences (Figure 9).

Hunt et al[11] have reported the sequence of the mMCP-9 cDNA of BALB/c strain. We sequenced the mMCP-9 cDNA obtained from CMCs of B6 strain. When we compared the sequence of mMCP-9 cDNA between BALB/c and B6 strains, five nucleotides (nucleotides +158, +202, +204, +258, and +493) were different, but one of five nucleotide changes did not result in the alternation of amino acids. The remaining four nucleotide changes caused the alternation of three amino acids: TTC codon at nucleotides 157 to 159 changed to TCC, leading to an alternation from Phe to Ser; GCC codon at nucleotides 202 to 204 changed to ACG, leading to an alternation from Ala to Thr; GTG codon at nucleotides 493 to 495 changed to TTG, leading to an alternation from Val to Leu (data not shown). When we compared the nucleotide sequence between WB and BALB/c strains, the same result was obtained as in the case of B6 strain (data not shown).

We compared the nucleotide sequences of mMCP-2, -4, and -9 between B6 and WB strains. Nine nucleotide changes and the resulting five amino acid alternations were found in the sequence of mMCP-2. Eleven nucleotide changes and the resulting three amino acid alternations were observed in mMCP-4, moreover deletion of 11 nucleotides were observed in the mMCP-4 sequence of the B6 strain. There were no differences in the sequence of mMCP-9 between B6 and WB strains. No particular stop codons were found in mMCP-2, -4, and -9 sequences of B6 strain. In fact, the size of the transcripts were comparable between B6 and WB strains (Figures 8 and 9).

Xia et al[47] reported that the mMCP-1, -2, and -4 transcripts have multiple UGXCCCC sequences in their 3′-UTRs. A possibility has been raised that these sequences may be the *cis*-acting elements that regulate the steady-state levels of these transcripts.[47] We found three UGXCCCC sequences in the 3′-UTR of mMCP-2 in all BALB/c, B6, and WB strains (Figure 8). In the 3′-UTR of mMCP-4, six UGXCCCC sequences were present in WB and DBA/2 strains, but only three UGXCCCC sequences in B6 strain (Figure 9).

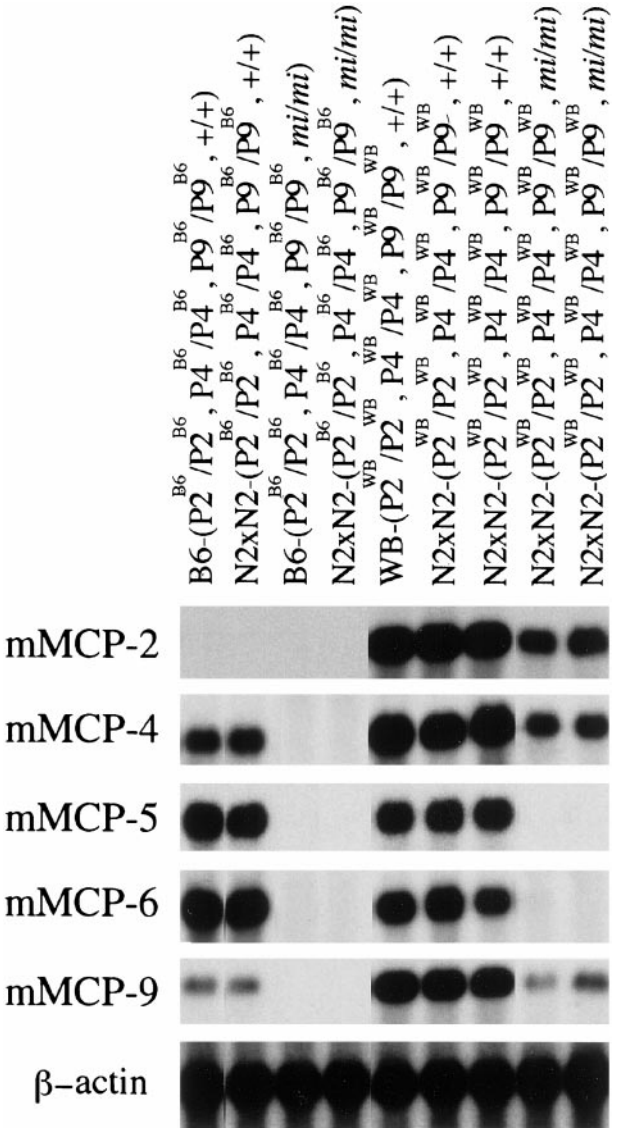


**Figure 10.** Expression of mMCP-2, -4, -5, -6, and -9 mRNA transcripts in CMCs of a B6-($P2^{B6}/P2^{B6}$, $P4^{B6}/P4^{B6}$, $P9^{B6}/P9^{B6}$, +/+) mouse, a N2 × N2-($P2^{B6}/P2^{B6}$, $P4^{B6}/P4^{B6}$, $P9^{B6}/P9^{B6}$, +/+) mouse, a B6- ($P2^{B6}/P2^{B6}$, $P4^{B6}/P4^{B6}$, $P9^{B6}/P9^{B6}$, *mi/mi*) mouse, a N2 × N2-($P2^{B6}/P2^{B6}$, $P4^{B6}/P4^{B6}$, $P9^{B6}/P9^{B6}$, *mi/mi*) mouse, a WB-($P2^{WB}/P2^{WB}$, $P4^{WB}/P4^{WB}$, $P9^{WB}/P9^{WB}$, +/+) mouse, two N2 × N2-($P2^{WB}/P2^{WB}$, $P4^{WB}/P4^{WB}$, $P9^{WB}/P9^{WB}$, +/+) mice, and two N2 × N2-($P2^{WB}/P2^{WB}$, $P4^{WB}/P4^{WB}$, $P9^{WB}/P9^{WB}$, *mi/mi*) mice. Total RNA was extracted from CMCs of each genotype, and 20 μg of total RNA was electrophoresed and hybridized with mMCP-2, -4, -5, -6, and -9 probes. Three similar experiments were done, and a representative result is shown. Comparable results were obtained in two other experiments.

### *Effect of MITF*

To examine the effect of MITF on the expression of the WB strain-derived mMCP-2, -4, and -9 genes, we crossed WB-($P2^{WB}/P2^{WB}$, $P4^{WB}/P4^{WB}$, $P9^{WB}/P9^{WB}$, +/+) mice to B6-($P2^{B6}/P2^{B6}$, $P4^{B6}/P4^{B6}$, $P9^{B6}/P9^{B6}$, *mi/+)* mice, and ultimately obtained mice of the following genotypes; ($P2^{B6}/P2^{B6}$, $P4^{B6}/P4^{B6}$, $P9^{B6}/P9^{B6}$, +/+), ($P2^{B6}/P2^{B6}$, $P4^{B6}/P4^{B6}$, $P9^{B6}/P9^{B6}$, *mi/mi),* ($P2^{WB}/P2^{WB}$, $P4^{WB}/P4^{WB}$, $P9^{WB}/P9^{WB}$, +/+) and ($P2^{WB}/P2^{WB}$, $P4^{WB}/P4^{WB}$, $P9^{WB}/P9^{WB}$, *mi/mi*). The strain origin of mMCP-2 and -4 genes was confirmed by sequencing cDNAs of mMCP-2 and -4 genes. Because there was no difference between the B6 and WB strain in the mMCP-9 cDNA, we confirmed the strain origin by sequencing the genomic DNAs of mMCP-9 genes (nucleotides +1617 to +2466). Because the expression of mMCP-5 and -6 genes was comparable between CMCs of WB-+/+ mice and those of B6-+/+ mice, we did not examine the strain origin of mMCP-5 and -6 genes. As already described, the expression of mMCP-2, -4, -5, -6, and -9 genes was reduced in B6-*mi/mi* mice when compared to B6-+/+ mice (Figure 1). The reduction of the similar magnitude was observed in CMCs derived from N2 × N2-($P2^{B6}/P2^{B6}$, $P4^{B6}/P4^{B6}$, $P9^{B6}/P9^{B6}$, *mi/mi*) mice (Figure 10). The expression of

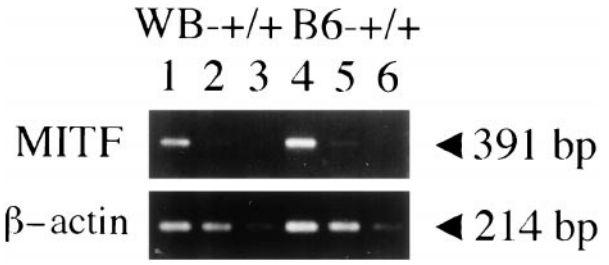


**Figure 11.** Semiquantitative RT-PCR analysis of expression of MITF mRNA in CMCs of WB-+/+ and B6-+/+ mouse origin. PCR products from RNAs of WB-+/+ CMCs (**lanes 1** to **3**) and from B6-+/+ CMCs (**lanes 4** to **6**) were electrophoresed in 1.0% agarose gel containing ethidium bromide. The amounts of RNA used for reverse transcription were 5 $\mu$g (**lanes 1** and **4**), 0.5 $\mu$g (**lanes 2** and **5**), and 0.05 $\mu$g (**lanes 3** and **6**), respectively. Three similar experiments were done, and a representative result is shown. Comparable results were obtained in two other experiments.

mMCP-2, -4, -5, -6, and -9 genes was also comparable between WB-+/+ and N2 × N2-(*P2*$^{WB}$/*P2*$^{WB}$, *P4*$^{WB}$/*P4*$^{WB}$, *P9*$^{WB}$/*P9*$^{WB}$, +/+) mice (Figure 10). When the expression of mMCP-2, -4, -5, -6, and -9 genes was compared between N2 × N2-(*P2*$^{WB}$/*P2*$^{WB}$, *P4*$^{WB}$/*P4*$^{WB}$, *P9*$^{WB}$/*P9*$^{WB}$, +/+) mice and N2 × N2-(*P2*$^{WB}$/*P2*$^{WB}$, *P4*$^{WB}$/*P4*$^{WB}$, *P9*$^{WB}$/*P9*$^{WB}$, *mi/mi*) mice, the expression of each protease gene was significantly greater in the former mice than in the latter mice (Figure 10). When the magnitude of reduction was compared among different proteases, it was greater in mMCP-5 and -6 genes than in mMCP-2, -4, and -9 genes (Figure 10).

We compared the expression of MITF between WB-+/+ and B6-+/+ CMCs using semiquantitative RT-PCR. The expression levels of MITF gene were comparable between WB-+/+ CMCs and B6-+/+ CMCs (Figure 11).

We examined whether the CANNTG motifs in the promoter of mMCP-2, -4, and -9 genes were conserved between B6 and WB strains by sequencing the 5′ flanking region of mMCP-2, -4, and -9 genes. All of the CACATG motif (nucleotides −377 to −372) of the mMCP-2 promoter, the CATGTG motif (nucleotides −383 to −378) of the mMCP-4 promoter[22] and CATATG motif (nucleotides −381 to −376) of the mMCP-9 promoter were conserved completely (data not shown).

## *Discussion*

The mRNA expression of the mMCP-2 and -9 genes was significantly higher in CMCs of B6-+/+ mice than in CMCs of B6-*mi/mi* mice. Overexpression of +-MITF but not of *mi*-MITF normalized the expression of mMCP-2 and -9 genes in CMCs of B6-*mi/mi* mice to the level of CMCs of B6-+/+ mice. When the luciferase construct containing the mMCP-2 promoter with the CACATG motif (nucleotides −377 to −372) was transfected into the IC-2 mast cell line, luciferase activity was enhanced. The transcription activity was abolished when the CACATG motif was deleted or mutated. GST + MITF fusion protein bound the CACATG motif in the 5′-flanking region of the mMCP-2 gene. In the case of mMCP-9 gene, luciferase activity was enhanced through the mMCP-9 promoter with the CATATG motif (nucleotides −381 to −376). The mutation or deletion of the CATATG motif abolished the transcription activity. Specific binding of +-MITF to the CATATG motif was demonstrated by EGMSA. These results suggested that +-MITF transactivated the mMCP-2 and -9 genes through, at least in part, the direct binding to the CANNTG motifs.

All mMCP-2, -4, -5, and -9 are chymases.[3–5,7,11] +-MITF directly bound CANNTG motifs in the promoter region of the mMCP-2, -4, and -9 genes and transactivated each gene,[22] whereas the binding of +-MITF to the CANNTG motif in the promoter region of the mMCP-5 gene was not detectable.[21] The +-MITF seemed to regulate the transactivation of the mMCP-5 gene indirectly. The regulation mechanism of mMCP-2, -4, and -9 genes seemed to be different from that of mMCP-5 gene.

The expression of mMCP-2, -4, and -9 genes was greater in CMCs of WB-+/+ mice than in those of B6-+/+ mice. This is consistent with the results of Eklund et al.[27] The poor expression of mMCP-7 gene in the B6 mice is because of the presence of a stop codon. The resulting truncated transcript degraded quickly in CMCs.[46] We compared the nucleotide sequences of mMCP-2, -4, and -9 cDNAs between WB and B6 strains, but no stop codons were observed in the sequences of mMCP-2, -4, and -9 cDNAs of B6 mice. The poor expression of mMCP-2, -4, and -9 genes was not considered to be because of the presence of stop codon in each cDNA of the B6 strain.

Multiple UGXCCCC sequences were found in the 3′UTR of mMCP-2 and -4 transcripts as reported by Xia et al.[47] Three UGXCCCC sequences were observed in 3′UTR of mMCP-2 in both B6 and WB strains. Three UGXCCCC sequences were found in 3′UTR of B6 mMCP-4 whereas six sequences in 3′UTR of WB mMCP-4. The smaller number of UGXCCCC sequences in the B6 mMCP-4 may explain the poor expression of mMCP-4 gene in CMCs of B6 strain. However, the rank order in difference of the expression between WB and B6 strains was mMCP-2 > mMCP-4, the poor expression of the B6 strain was not completely explained by the possible effect of the repetitive sequences on the stability of $\beta$-chymases.

The expression of mMCP-2, -4, and -9 genes was influenced by the strain difference between B6 and WB mice, but the expression of mMCP-5 and -6 genes was comparable between B6 and WB mice. Although mMCP-5 is a chymase as mMCP-2, -4, and -9, the regulation of mMCP-5 gene was similar to that of mMCP-6 gene encoding a tryptase rather than to that of mMCP-2, -4, and -9 genes. This is consistent with the result of Morii et al.[21] All mMCP-2, -4, -9, and -5 genes were located on chromosome 14, but the regulation of mMCP-5 seemed to be unique among chymases.

Our observation that the regulation of mMCP-5 was different from that of mMCP-2, -4, and -9 is consistent with the reported characteristics of mMCP-5.[48] mMCP-5 is the only mouse chymase which has a close human

homologue.[49] Although α- and β-chymases are known in mammalian mast cells, human, baboon, and dog mast cells contain only α-chymases.[5,49–53] In contrast, mast cells of mice, rat, and gerbils have been reported to contain four, two, and one β chymase-encoding genes, respectively.[50,53] All mMCP-2, -4, and -9 are β-chymases, and the transcriptional regulation of mMCP-2, -4, and -9 genes is highly similar.[48,49] The transcriptional behavior of the mMCP-5 gene may resemble to that of genes encoding other α-chymases.

We examined the effect of mutation of the *mi* locus on the expression of WB strain-derived mMCP-2, -4, and -9 genes. The expression of these genes was reduced in N2 × N2-($P2^{WB}/P2^{WB}$, $P4^{WB}/P4^{WB}$, $P9^{WB}/P9^{WB}$, *mi/mi*) mice when compared to that of N2 × N2-($P2^{WB}/P2^{WB}$, $P4^{WB}/P4^{WB}$, $P9^{WB}/P9^{WB}$, +/+) mice. The *mi* mutation affected not only the B6 strain-derived mMCP-2, -4, and -9 genes but also the WB strain-derived counterparts. The expression level of MITF was comparable between CMCs of WB-+/+ mice and CMCs of B6-+/+ mice. Moreover, the CANNTG motifs in the promoter region of mMCP-2, -4, and -9 genes were not different between B6 and WB strains. Therefore, the interstrain difference in the expression of mMCP-2, -4, and -9 genes was not attributable to the interaction of MITF and each CANNTG motifs.

The rank order of the magnitude in the expression of mMCP-2 gene was ($P2^{WB}/P2^{WB}$, +/+) > ($P2^{WB}/P2^{WB}$, *mi/mi*) > ($P2^{B6}/P2^{B6}$, +/+) > ($P2^{B6}/P2^{B6}$, *mi/mi*). That of mMCP-4 gene was ($P4^{WB}/P4^{WB}$, +/+) > ($P4^{WB}/P4^{WB}$, *mi/mi*) ~ ($P4^{B6}/P4^{B6}$, +/+) > ($P4^{B6}/P4^{B6}$, *mi/mi*). That of mMCP-9 gene was ($P9^{WB}/P9^{WB}$, +/+) > ($P9^{WB}/P9^{WB}$, *mi/mi*) ~ ($P9^{B6}/P9^{B6}$, +/+) > ($P9^{B6}/P9^{B6}$, *mi/mi*). This suggested that the effect of the strain difference of particular mMCP genes and the effect of *mi* mutation were independent of each other, and that both effects were additive on the expression of particular mMCP genes.

All genes encoding granzyme B, cathepsin G, mMCP-1, -2, -4, -5, and -9 are located on chromosome 14.[12] It was suggested that there may be a locus control region for the granzymes and another locus control region for cathepsin G and the downstream chymases.[54,55] The expression of mMCP-2, -4, and -9 genes was greater in CMCs of WB-+/+ mice than in CMCs of B6-+/+ mice. The higher expression of these chymase genes in CMCs of WB-+/+ mice may be driven by the locus control region for cathepsin G and the downstream chymases. The rank order in difference of the expression between WB and B6 strains was mMCP-2 > mMCP-9 > mMCP-4. It was reported that mMCP-2 was mapped ~30 kb downstream from the cathepsin G gene.[55] Because the effect of locus control region activity is considered to be distance-dependent,[56–58] the rank order might reflect on the distance from the locus control region.

Reed et al[59] examined whether the strain of mouse affected the mast cell development. Bone marrow cells taken from SWR and NIH mice produced large numbers of CMCs whereas those from C57BL/10 mice produced relatively few numbers of CMCs. The result suggested that development of mast cell was strain-dependent. We showed that the expression of mMCP genes was also strain-dependent. Studying the expression of mMCP genes seems to be a good model for clarifying the strain-dependent mast cell differentiation.

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