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# Cell Type-Specific Deficiency of c-*kit* Gene Expression in Mutant Mice of *mi/mi* Genotype

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***The* mi *locus of mice encodes a novel member of the basic-helix-loop-helix-leucine zipper protein family of transcription factors (hereafter called* mi *factor). In addition to microphthalmus, osteopetrosis, and lack of melanocytes, mice of* mi/mi *genotype are deficient in mast cells. Since the c-*kit *receptor tyrosine kinase plays an important role in the development of mast cells, and since the c-*kit *expression by cultured mast cells from* mi/mi *mice is deficient in both mRNA and protein levels, the mast cell deficiency of* mi/mi *mice has been attributed at least in part to the deficient expression of c-*kit*. However, it remained to be examined whether the c-*kit *expression was also deficient in tissues of* mi/mi *mice. In the present study, we examined the c-*kit *expression by* mi/mi *skin mast cells using* in situ *hybridization and immunohistochemistry. Moreover, we examined the c-*kit *expression by various cells other than mast cells in tissues of* mi/mi *mice. We found that the c-*kit *expression was deficient in mast cells but not in erythroid precursors, testicular germ cells, and neurons of* mi/mi *mice. This suggested that the regulation of the c-*kit *transcription by the* mi *factor was dependent on cell types. Mice of* mi/mi *genotype appeared to be a useful model to analyze the function of transcription factors in the whole-animal level. (Am J Pathol 1994, 145:827–836)***

A double gene dose of mutant alleles at the *mi* locus on chromosome 6 produces the pleiotrophic effects of microphthalmus, depletion of pigment in both hair and eyes, and osteopetrosis.[1,2] In addition to these abnormalities, Stevens and Loutit[3] and Stechshulte et al[4] reported depletion of mast cells in *mi/mi* mice. However, when compared with the mast cell depletion of $W/W^v$ and $Sl/Sl^d$ mice, that of *mi/mi* mice is moderate.[5–7] We studied the mechanism of the mast cell depletion of *mi/mi* mice.[7] Cultured mast cells (CMCs) derived from the spleens of *mi/mi* mice continued to proliferate when T-cell-derived growth factors were supplied, but *mi/mi* CMCs were not maintained in the co-culture with the NIH/3T3 fibroblasts which produced stem cell factor (SCF).[7] We also demonstrated that the expression of c-*kit* receptor tyrosine kinase was much less in *mi/mi* CMCs in both mRNA and protein levels and that *mi/mi* CMCs did not respond to SCF that is a ligand for c-*kit*.[8]

The *W* locus encodes the c-*kit* receptor tyrosine kinase,[9,10] whereas the *Sl* locus encodes SCF.[11–14] Recently, the *mi* locus was shown to encode a novel number of the basic-helix-loop-helix-leucine zipper (bHLH-ZIP) protein family of transcription factors.[15,16] In addition to the decrease in number of mast cells, the phenotype of mast cells in the skin of *mi/mi* mice was abnormal.[7,8,17] Approximately one-half of mast cells in the skin of +/+ and $W/W^v$ mice expressed the mRNA of mouse mast cell protease 6 (MMCP-6), but only 3% of mast cells expressed MMCP-6 mRNA in the skin of *mi/mi* mice.[17] The expression of MMCP-6 was markedly reduced in CMCs of *mi/mi* genotype as well.[8] There is a possibility that the transcription of the c-*kit* and MMCP-6 genes may be regulated by the transcription factor encoded by the *mi* locus.

The deficiencies of melanocytes and mast cells are common abnormalities in the homozygous or double heterozygous mutants at the *mi*, *W*, or *Sl* locus.[1–7,18] In addition to deficiencies of mast cells and melanocytes, deficiencies of erythrocytes and germ cells are observed in $W/W^v$ and $Sl/Sl^d$ mice.[1,18] Although *mi/mi* mice are not deficient in erythrocytes and germ cells, they show microphthalmus and osteopetrosis.[1,2,4]

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Since the c-*kit* receptor is expressed by mast cells,[19–23] melanocytes,[23–27] erythroid cells,[23,28–31] germ cells[23–26,32,33] and/or their precursors, the depletion of mast cells and melanocytes in *mi/mi* mice may be attributed to the deficient expression of the c-*kit*. We have already demonstrated the deficient expression of c-*kit* by CMCs of *mi/mi* genotype,[8] but it remains to be examined whether the expression of c-*kit* by mast cells is also deficient in tissues of *mi/mi* mice. In the present study, we examined the c-*kit* expression by *mi/mi* skin mast cells in both mRNA and protein levels. Moreover, we examined the c-*kit* expression by various cells other than mast cells in tissues of *mi/mi* mice. We found that the c-*kit* expression was deficient in mast cells but not in erythroid precursors, testicular germ cells, or neurons of *mi/mi* mice.

## Materials and Methods

### Mice and Embryos

The original stock of C57BL/6-*mi*/+ (*mi*/+) mice was purchased from the Jackson Laboratory, Bar Harbor, ME, and the mice were maintained in our laboratory by repeated backcrosses to C57BL/6 mice of our own inbred colony. Mice of *mi*/+ genotype were mated, and the resulting *mi/mi* embryos or suckling mice were selected by microphthalmus and/or white coat color. C57BL/6-+/+ (+/+) mice were mated to obtain +/+ embryos and suckling mice. For embryonic staging, the day of vaginal plug observation was considered as day 0 postcoitum (p.c.).

### Numbers of Erythrocytes and Mast Cells

Pregnant mice of 18 days p.c. were killed by overdose of ether and embryos were removed. Blood samples from the embryos and 20-day-old mice were obtained by decapitation under ether anesthesia. The number of erythrocytes was counted with a hemocytometer. Skin pieces were removed from the backs of 20-day-old mice and smoothed onto a piece of filter paper to keep them flat. The embryos and skin pieces were fixed in Carnoy's solution and embedded in paraffin. Sections (4 μ thick) were stained with Alcian blue and nuclear fast red. In the sagittal section of embryos, two points were marked in ink on the back, one near the head and another near the tail; mast cells in the subcutaneous connective tissue between these two points were counted under the microscope. In the section of skin pieces, mast cells between epithelium and panniculus carnosus were counted. The number of mast cells thus obtained in the embryo and skin was divided by the length of the portion in which mast cells were counted and expressed as mast cells per centimeter.[5,34]

### Assay of Colony-Forming Units in Spleen

Bone marrow cells of 20-day-old *mi/mi* and +/+ mice were obtained as described.[35] Recipient C57BL/6-+/+ mice received 8.0 Gy whole-body irradiation with a Rigaku Radioflex 350 X-ray apparatus. Bone marrow cells ($5 \times 10^4$) suspended in 0.2 ml Eagle's medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) were injected into the lateral tail vein of recipients within 3 hours after the irradiation. The recipients were killed by overdose of ether 8 days after the injection of bone marrow cells. Spleens were removed and fixed in Bouin's fluid. The number of spleen colonies was counted under the dissection microscope (×10).

### Immunohistochemistry

The ACK2 rat monoclonal antibody (MAb) against the extracellular domain of the murine c-*kit* receptor was a generous gift from Dr. S.-I. Nishikawa.[27,30] Freshly dissected tissue samples were covered with Tissue-Tek OCT compound (an embedding medium to freeze tissues; Miles Inc., Elkhart, IN) and quickly frozen in liquid nitrogen; frozen sections (4 μ thick) were prepared with a cryostat. The sections were fixed with ice-cold methanol-acetone (1:1), washed in phosphate-buffered saline (PBS, pH 7.5), and incubated with ACK2 MAb at the concentration of 10 μg/ml in PBS overnight at 4 C. Then the sections were washed five times with PBS and incubated with FITC-conjugated rabbit anti-rat IgG antibody (DACO A/S, Glostrup, Denmark) for 40 minutes at 4 C, followed by extensive washing with PBS. The specimens were examined with a confocal laser scanning microscope (LSM-GM200, Olympus, Tokyo, Japan). After examination with the confocal laser scanning microscope, the specimens were washed with distilled water and stained with Alcian blue and nuclear fast red to confirm the location of mast cells.

To count c-*kit* protein$^+$ cells in the skin, fetal liver, and testis and to examine c-*kit* protein$^+$ cells in the hair follicles, dorsal root ganglion (DRG), cerebellum, the binding of ACK2 MAb was visualized with streptavidin-biotin-peroxidase and 0.05% diaminobenzidine-0.02% $H_2O_2$ solution (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's instructions. The sections were counterstained with hematoxylin. The method to

count mast cells has been mentioned above. The adjacent cryostat sections were stained with Alcian blue and nuclear fast red, and the number of Alcian blue$^+$ cells was counted to calculate the proportion of c-*kit* protein$^+$ cells to Alcian blue$^+$ cells. In the fetal liver, c-*kit* protein$^+$ cells were counted under the microscope with a square micrometer attached to eyepiece and expressed as number of cells per mm$^2$. In the testis, c-*kit* protein$^+$ cells were also counted under the microscope and expressed as number of cells per cross-section of a seminiferous tubule.[32]

## *Preparation of RNA Probes*

The following complementary (c) DNA clones were used as hybridization probes: mouse c-*kit* cDNA containing a 4244-bp fragment (1 to 4244)[36] and mouse mast cell carboxypeptidase A (MC-CPA) cDNA containing a 690-bp fragment (688 to 1377).[37] For generation of mouse c-*kit* probes, five fragments (1 to 1064, 1065 to 1620, 1621 to 2548, 2549 to 3564, and 3565 to 4244) of mouse c-*kit* cDNA (a generous gift of Dr. S.-I. Nishikawa) were subcloned into pBluescript KS −. Mouse MC-CPA cDNA was prepared by reverse transcription of mRNA obtained from CMCs of +/+ mice, followed by polymerase chain reaction. This cDNA fragment (688 to 1377) was subcloned into pBluescript KS −. DNA sequencing was performed by the method of Sanger et al[38] using [$^{32}$P]-$\alpha$-deoxycytidine triphosphate (DuPont/NEN Research Products, Boston, MA).

## In Situ *Hybridization*

Details of the *in situ* hybridization technique have been described previously.[39] Digoxigenin-labeled single-strand RNA probes were prepared for hybridization using a DIG RNA labeling kit (Boehringer Mannheim Biochemica, Mannheim, Germany) according to the manufacturer's instructions. Hybridization of c-*kit* and MC-CPA mRNAs was performed at 50 C for 16 hours, and the signals were detected using a nucleic acid detection kit (Boehringer). The controls included: 1) hybridization with the sense probes, 2) RNAse treatment before hybridization, and 3) use of neither the antisense RNA probe nor the antidigoxygenin antibody.

## *L-3,4-Dihydroxyphenylalanine (L-DOPA) Reaction*

For demonstration of melanocytes in embryos, melanin synthesis from L-DOPA (Sigma Chemical Co., St. Louis, MO) was examined histochemically.[40] Embryos were fixed in ice-cold 2% calcium acetate-4% paraformaldehyde solution. After washing with distilled water, they were incubated in 100 mmol/L phosphate buffer (PB, pH 7.0) containing 0.1% L-DOPA 20 C overnight. They were washed with distilled water, dehydrated with ethanol series and embedded in paraffin. Sections (4 μ thick) were counterstained with nuclear fast red.

## *Immunoprecipitation Analysis*

To obtain CMCs, spleen cells were cultured in the presence of pokeweed mitogen-stimulated spleen cell-conditioned medium (PWM-SCM) as described previously.[41] Testes were removed from 20-day-old mice and were decapsulated. Seminiferous tubules were unraveled by forceps and gentle pipetting in ice-cold PBS. The dispersed tubules were allowed to sediment, and supernatant was filtered through a nylon mesh and collected as testicular cells.

For metabolic labeling, $4 \times 10^6$ CMCs and $5 \times 10^7$ testicular cells were washed with pre-warmed PBS and incubated for 2 hours in methionine-free Eagle's minimal essential medium containing 5 mmol/L glutamine, 1 mmol/L sodium pyruvate, and 10% dialyzed fetal bovine serum (Hazleton, Lenexa, KS). Incubation temperatures were 37 C and 32.5 C in CMCs and testicular cells, respectively. After the medium change, [$^{35}$S] methionine (DuPont/NEN; 100 μCi/ml in CMCs and 200 μCi/ml in testicular cells) was added to the fresh medium and incubation continued for 6 hours. The labeled cells were collected, washed with ice-cold PBS, and lysed in TDS buffer [10 mmol/L $Na_2HPO_4$ (pH 7.2) and 160 mmol/L NaCl containing 1% Triton X-100, 1% deoxycholic acid, and 0.3% sodium dodecyl sulfate (SDS)]. After 30 minutes on ice, the extracts were centrifuged at 50,000 rpm for 30 minutes at 4 C to remove insoluble cell debris. Lysates were precleared with protein G-Sepharose beads (Pharmacia, Uppsala, Sweden) and then precipitated with ACK2 MAb or class-matched control antibody conjugated to protein G-Sepharose beads in TDS buffer for 12 hours at 4 C. Immunoprecipitates were washed with TDS buffer three times and with 10 mmol/L PB containing 0.5% Tween 20 four times, resuspended in sample buffer [10 mmol/L Tris-HCl (pH 7.4) containing 4% SDS, 10% $\beta$-mercaptoethanol, and 10% glycerol], and boiled for 5 minutes. Electrophoretical separation was performed on 7.5% SDS-polyacrylamide gels, according to the method of Laemmli.[42] Gels were fixed with 10% acetic acid and 30% methanol and treated with 1 mol/L sodium salicylate before autoradiography.

## Results

The numbers of mast cells, erythrocytes, and colony-forming units in spleen (CFU-S) were compared between *mi/mi* and control +/+ embryos of day-18 p.c. and between *mi/mi* and control +/+ mice of 20 days of age. In both day-18 p.c. *mi/mi* embryos and 20-day-old *mi/mi* mice, the number of mast cells was apparently reduced, but the difference between the *mi/mi* and +/+ mice was greater in day-18 p.c. embryos than in 20-day-old mice (Table 1). In contrast, the numbers of erythrocytes in day-18 p.c. *mi/mi* embryos and 20-day-old *mi/mi* mice were comparable with those of control +/+ embryos and mice. The number of CFU-S was normal in the bone marrow of 20-day-old *mi/mi* mice (Table 1). Moreover, the size of spleen colonies produced by *mi/mi* CFU-S was comparable with that of spleen colonies produced by +/+ CFU-S.

The expression of c-*kit* mRNA was examined in the skin of day-18 p.c. embryos by *in situ* hybridization. As a control, the expression of MC-CPA was examined using the same samples. Numbers of mast cells were counted in the adjacent sections stained with Alcian blue and nuclear fast red, and proportions of MC-CPA mRNA$^+$ or c-*kit* mRNA$^+$ mast cells to Alcian blue-positive mast cells were calculated. Most mast cells in the skin of both +/+ and *mi/mi* embryos expressed MC-CPA mRNA (Table 2 and Figure 1, A to D). Approximately one-half of mast cells in the skin of +/+ embryos expressed the c-*kit* mRNA, whereas only 1% of mast cells expressed it in the skin of *mi/mi* embryos (Table 2 and Figure 1, E to H). We also examined the expression of the c-*kit* mRNA in *mi/mi* and control +/+ mice of 20 days of age. Since the proportion of c-*kit* mRNA$^+$ cells decreased in the skin of +/+ mice, the difference between *mi/mi* and +/+ mice was reduced (Table 2). There is a possibility that the immunohistochemistry is more sensitive than the *in situ* hybridization to demonstrate the expression of the c-*kit*. We stained skin mast cells with ACK2 MAb that binds the extracellular domain of the c-*kit*. Most mast cells in the skin of day-18 p.c. +/+ embryos and 20-day-old +/+ mice were stained with ACK2 MAb, but only 12% of mast cells in the skin of day-18 p.c. *mi/mi* embryos and 37% of mast cells in the skin of 20-day-old *mi/mi* mice were stained by ACK2 MAb (Table 2). The intensity of binding with ACK2 MAb was evaluated using confocal laser scanning microscopy; some mast cells in the skin of day-18 p.c. +/+ embryos and 20-day-old +/+ mice showed strong fluorescence. In contrast, mast cells showing significant fluorescence was rare in the skin of day-18 p.c. *mi/mi* embryos (Figure 2). Mast cells in the skin of 20-day-old *mi/mi* mice showed the fluorescent intensity of apparently detectable level, but the intensity was weaker when compared with that of control +/+ mast cells (Figure 2).

Both precursors of mast cells and those of melanocytes migrate and differentiate within the skin.[23–27,43] In skin tissues of day-18 p.c. *mi/mi* embryos, we detected very few c-*kit* protein$^+$ cells. Most of those c-*kit* protein$^+$ cells were Alcian blue$^+$ mast cells. Therefore, practically no c-*kit*-expressing melanoblasts were detectable in the skin tissues of day-18 p.c. *mi/mi* embryos. Figure 3, A to D, shows developing hair follicles in the skin of +/+ and *mi/mi* embryos of day-18 p.c. The c-*kit* expressing melanocytes were detectable only in the hair follicle of +/+ embryos.

Both mast cells and erythrocytes are derived from CFU-S.[44,45] Since the number of erythrocytes was normal in day-18 p.c. *mi/mi* embryos and 20-day-old *mi/mi* mice, we histochemically examined the expression of c-*kit* protein in the liver of day-18 p.c. *mi/mi* embryos. Some hematopoietic cells in the liver of day-18 p.c. *mi/mi* embryos expressed c-*kit* (Figure 3E). The number of c-*kit* protein$^+$ cells per unit area of the fetal liver was comparable between *mi/mi* and control +/+ embryos (Table 3).

Both melanocytes and DRG neurons are derived from the neural crest.[46] In spite of the lack of melanocytes in *mi/mi* embryos, some DRG neurons of

**Table 1.** *Numbers of Mast Cells, Erythrocytes, and CFU-S in Embryos and Mice of* +/+ *or* mi/mi *Genotype*

| Mice | Genotype | Number of cells ± SE: Mast cells (per cm skin) | Erythrocytes (per liter × $10^{-12}$) | CFU-S (per $10^5$ bone marrow cells) |
|---|---|---|---|---|
| Day-18 p.c. embryos | +/+ | 402 ± 12 (9) | 3.00 ± 0.04 (9) | NE |
| Day-18 p.c. embryos | *mi/mi* | 28 ± 2* (9) | 2.98 ± 0.05 (9) | NE |
| 20-day-old mice | +/+ | 321 ± 20† (7) | 7.61 ± 0.18 (7) | 13.4 ± 0.6 (11) |
| 20-day-old mice | *mi/mi* | 112 ± 8*† (7) | 7.35 ± 0.22 (7) | 11.4 ± 0.6 (11) |

Number of mice is shown in parentheses.
* $P < 0.01$ when compared with values of +/+ embryos or +/+ mice by *t* test.
† $P < 0.01$ when compared with values of embryos of the same genotype by *t* test.

Table 2. *Suppression of c-*kit *Expression in Mast Cells in the Skin of Embryos and Mice of* mi/mi *Genotype*

| Mice | Genotype | Proportion to Alcian blue$^+$ cells ± SE | | |
|---|---|---|---|---|
| | | MC-CPA mRNA$^+$ cells (%) | c-*kit* mRNA$^+$ cells (%) | c-*kit* protein$^+$ cells (%) |
| Day-18 p.c. embryos | +/+ | 89 ± 1 (5) | 47 ± 3 (5) | 99 ± 1 (6) |
| Day-18 p.c. embryos | *mi/mi* | 88 ± 5 (5) | 1 ± 1* (5) | 12 ± 2* (5) |
| 20-day-old mice | +/+ | 90 ± 3 (5) | 27 ± 4† (5) | 97 ± 1 (6) |
| 20-day-old mice | *mi/mi* | 88 ± 3 (5) | 3 ± 1* (5) | 37 ± 3*† (6) |

Number of mice is shown in parentheses.
* $P < 0.01$ when compared with values of +/+ embryos or +/+ mice by *t* test.
† $P < 0.01$ when compared with values of embryos of the same genotype by *t* test.

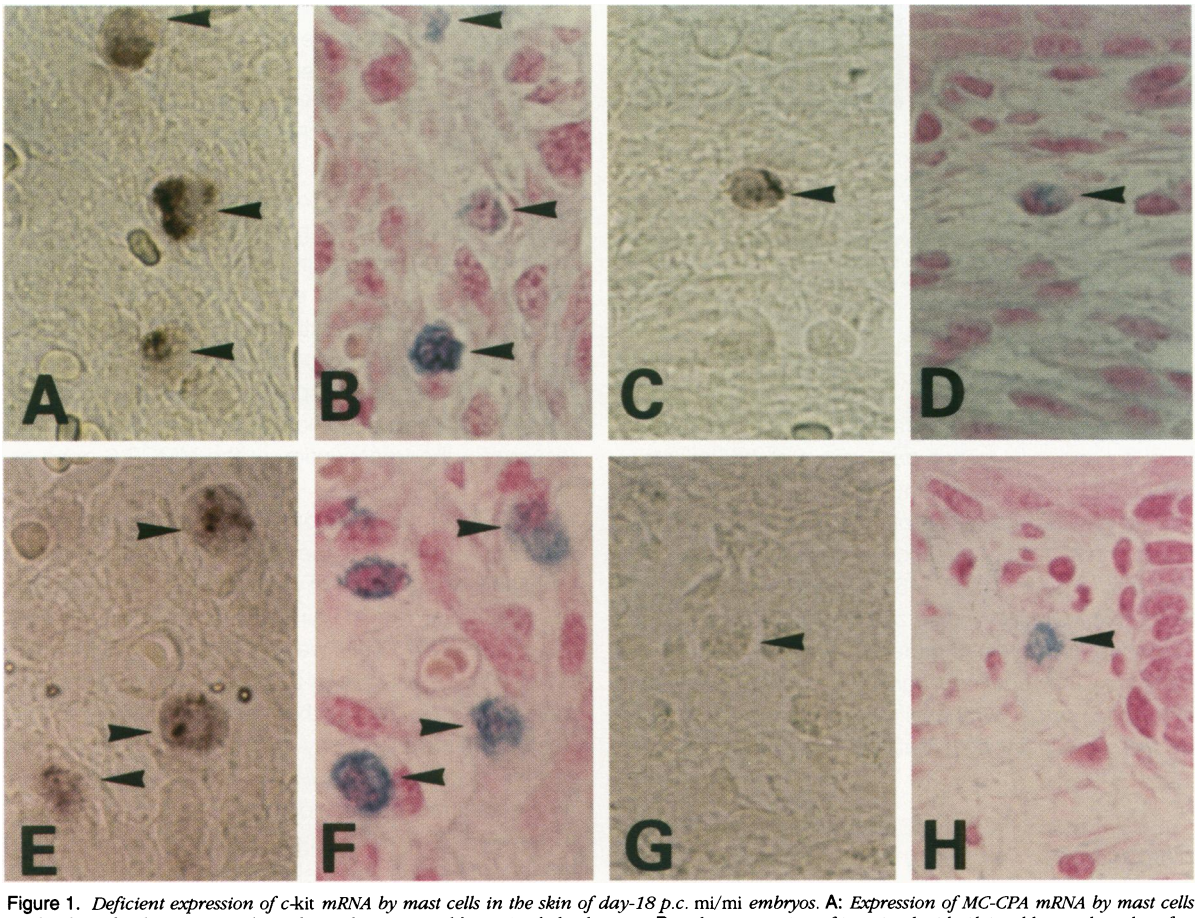


**Figure 1.** *Deficient expression of c-*kit *mRNA by mast cells in the skin of day-18 p.c.* mi/mi *embryos.* **A:** *Expression of MC-CPA mRNA by mast cells in the skin of a day-18 p.c. +/+ embryo, demonstrated by* in situ *hybridization.* **B:** *Adjacent section of* A *stained with Alcian blue and nuclear fast red, demonstrating mast cells.* **C:** *A MC-CPA mRNA$^+$ mast cell in the skin of a day-18 p.c.* mi/mi *embryo.* **D:** *Adjacent section of* C *stained with Alcian blue and nuclear fast red.* **E:** c-kit *mRNA$^+$ mast cells in the skin of the day-18 p.c. +/+ embryo.* **F:** *Adjacent section of* **E** *stained with Alcian blue and nuclear fast red.* **G:** *No c-*kit *mRNA$^+$ cells were observed in the skin of the 18-day p.c.* mi/mi *embryo.* **H:** *Adjacent section of* **G**, *demonstrating the presence of an Alcian blue$^+$ mast cell. Arrowheads indicate the same mast cells in the paired photographs.* × 760.

*mi/mi* embryos expressed c-*kit* protein (Figure 3F). Moreover, in the cerebellum of 20-day-old *mi/mi* mice, c-*kit* protein$^+$ basket cells were observed in the molecular layer, and c-*kit* protein$^+$ basket-like nerve endings (pinceau) around Purkinje cells (Figure 3G). The presence of c-*kit* mRNA was also demonstrated in basket cells in the molecular layer and in Golgi cells in the granular layer of the cerebellum by *in situ* hybridization (Figures 3H and 4).

The normal expression of c-*kit* mRNA was also detectable in spermatogonia and Leydig cells in the testis of 20-day-old *mi/mi* mice (Figure 5). As shown in Table 3, the number of c-*kit* protein$^+$ cells within the seminiferous tubules was comparable between *mi/mi* mice and control +/+ mice. Germ cells and Sertoli cells are present in the seminiferous tubules. Since only germ cells are c-*kit*$^+$,[26,32,33] we consider that the number shown in Table 3 represents the number of

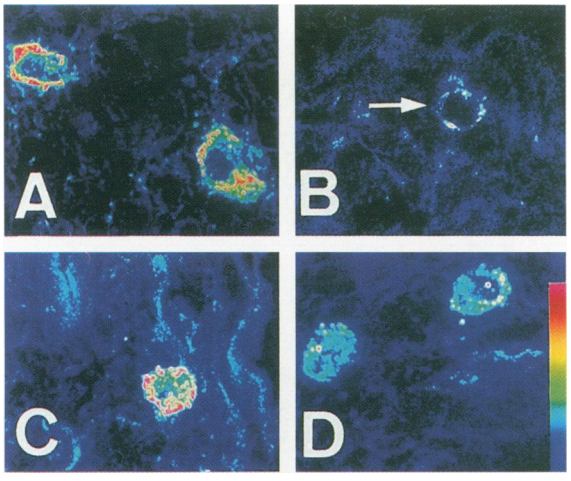


**Figure 2.** *Fluorescent intensity demonstrating the magnitude of binding with the ACK2 MAb. The photographs were taken with the Olympus confocal laser scanning microscope; red indicates a stronger fluorescent intensity than blue.* **A:** *Mast cells in the skin of a day-18 p.c. +/+ embryo.* **B:** *Mast cell in the skin of a day-18 p.c.* mi/mi *embryo. The cell shown by an arrow was confirmed to be a mast cell by staining the same section with Alcian blue and nuclear fast red.* **C:** *Mast cell in the skin of a 20-day-old +/+ mouse.* **D:** *Two mast cells in the skin of a 20-day-old* mi/mi *mouse.* × *760.*

c-*kit* protein$^+$ germ cells. Testicular cells and CMCs of *mi/mi* and +/+ genotypes were metabolically labeled with [$^{35}$S] methionine. The synthesis of both 145-kd and 125-kd c-*kit* protein was comparable between *mi/mi* and +/+ testicular cells. Although +/+ CMCs synthesized both 145-kd and 125-kd c-*kit* proteins, *mi/mi* CMCs synthesized only small amounts of 125-kd c-*kit* protein (Figure 6). Since 145-kd c-*kit* protein is the functional form,[20,21,47] Figure 6 indicated the normal synthesis of functional c-*kit* protein by *mi/mi* testicular cells but not by *mi/mi* CMCs.

## *Discussion*

The number of mast cells was much fewer in the skin of *mi/mi* embryos than that of +/+ embryos. The number of mast cells increased in *mi/mi* mice after birth, and the difference between *mi/mi* and +/+ mice decreased at 20 days of age. Although the proportion of c-*kit* mRNA$^+$ cells remained at 3% in the skin of 20-day-old *mi/mi* mice, the proportion of c-*kit* protein$^+$ cells increased up to 37%. This may explain at least in part the postnatal increase of mast cells in the skin of *mi/mi* mice. There is a possibility that the bHLH-ZIP transcription factor encoded by the *mi* locus[15,16] (hereafter called *mi* factor) regulates the transcription of the c-*kit* gene in an age-dependent manner. Since transcription factors other than *mi* factor may also regulate the c-*kit* gene expression, there is another possibility that the role of the other transcription factors might increase after birth.

Mast cells and erythrocytes are derived from CFU-S.[44,45] Although the number and the size of spleen colonies produced by *W* (c-*kit*) mutant animals significantly decreased,[48,49] these parameters of spleen colonies derived from *mi/mi* mice were normal. Therefore, CFU-S of *mi/mi* mice appeared to express the c-*kit* receptor tyrosine kinase normally. Since the proportion of c-*kit* protein$^+$ cells in the liver of *mi/mi* embryos was comparable with that of +/+ embryos, the normal expression of the c-*kit* appeared to continue when CFU-S differentiated into the erythroid lineage. On the other hand, the magnitude of the c-*kit* expression decreased when CFU-S of *mi/mi* genotype differentiated into the mast cell lineage. There is a possibility that the normal *mi* factor may enhance the transcription of the c-*kit* gene in mast cells but not in erythroid cells. As a result, the mutation at the *mi* locus may influence the c-*kit* expression in the mast cell lineage but not in the erythroid lineage. In both CMCs and skin mast cells of *mi/mi* mice, the expression of MMCP-6, a mast cell-specific protease, was deficient.[8,17] Probably, the normal *mi* factor may be necessary for the transcription of the MMCP-6 gene as well.

The signal transduction through the c-*kit* receptor tyrosine kinase is indispensable for migration, differentiation, and survival of melanocytes as in the case of mast cells.[23–27] In hair follicles of *mi/mi* embryos, no c-*kit* protein$^+$ cells were present and no melanocytes were detectable by L-DOPA reaction. Moreover, no c-*kit* protein$^+$ cells other than mast cells were detectable in the skin of day-18 p.c. *mi/mi* embryos. The lack of melanocytes in *mi/mi* mice may be attributable to the deficient expression of the c-*kit*. Both melanocytes and DRG neurons are derived from the neural crest.[46] In contrast to melanocytes, the proportion of c-*kit* protein$^+$ cells in *mi/mi* DRGs was comparable with that of +/+ DRGs. Recently, Hirata et al[50] induced the neurite outgrowth from the c-*kit* protein$^+$ DRG neurons by SCF, indicating that the c-*kit* receptor expressed on DRG neurons are functional. The present result suggested that the *mi* factor was necessary for the expression of the c-*kit* gene when neural crest cells differentiated into melanocytes, but not when they differentiated into DRG neurons.

The c-*kit* mRNA was normally expressed by testicular germ cells and Leydig cells of *mi/mi* mice. Both 125-kd and 145-kd c-*kit* proteins were produced by testicular cells of *mi/mi* mice, but only small amounts of 125-kd protein were produced by *mi/mi* CMCs.

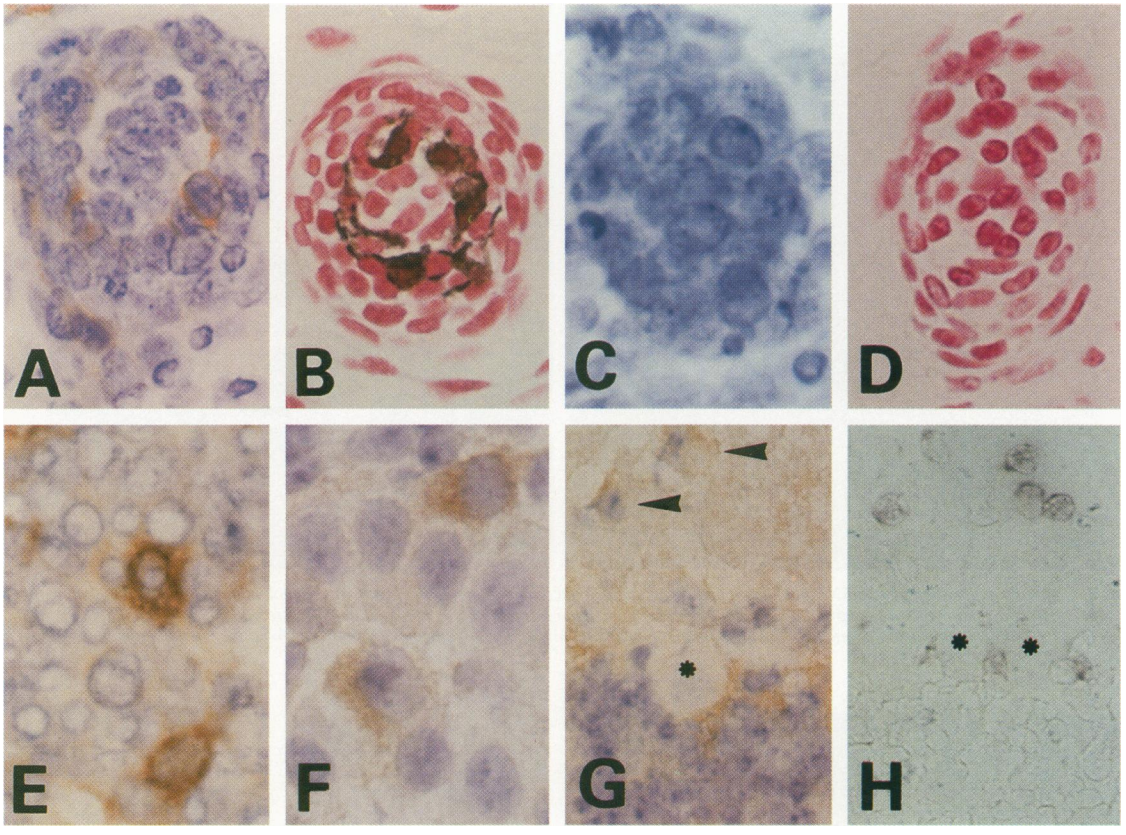


Figure 3. **A:** *Hair follicle of a day-18 p.c. +/+ embryo stained with ACK2 MAb, demonstrating the presence of* c-kit *protein*$^+$ *cells.* **B:** *Another hair follicle of the +/+ embryo after* L*-DOPA reaction, demonstrating the presence of melanocytes.* **C:** *Hair follicle of a day-18 p.c.* mi/mi *embryo stained with ACK2 MAb. No* c-kit *protein*$^+$ *cells were detectable.* **D:** *Another hair follicle of the* mi/mi *embryo after* L*-DOPA reaction. No melanocytes were observed.* **E:** *Liver of the day-18 p.c.* mi/mi *embryo stained with ACK2 MAb, demonstrating* c-kit *protein*$^+$ *hematopoietic cells.* **F:** *DRG of the* mi/mi *embryo stained with ACK2 MAb, demonstrating* c-kit *protein*$^+$ *neurons.* **G:** *Cerebellum of the* mi/mi *mice stained with ACK2 MAb, demonstrating* c-kit *protein*$^+$ *basket cells* (arrowheads) *and* c-kit *protein*$^+$ *basket-like nerve endings* (pinceau) *around a Purkinje cell* (asterisk). **H:** *c-kit mRNA*$^+$ *basket cells in the cerebellum of the* mi/mi *mice demonstrated by* in situ *hybridization.* Asterisks *indicate Purkinje cells.* × *760.*

Table 3. *Numbers of* c-kit *protein*$^+$ *Cells in the Fetal Livers and Testes of +/+ and* mi/mi *mice*

| Mice | Genotype | Tissue examined | No. of c-*kit* protein$^+$cells in unit area* |
|---|---|---|---|
| Day-18 p.c. embryos | +/+ | Liver | 427 ± 6 (10) |
| Day-18 p.c. embryos | *mi/mi* | Liver | 420 ± 6$^\dagger$ (5) |
| 20-day-old mice | +/+ | Testis | 20 ± 1 (10) |
| 20-day-old mice | *mi/mi* | Testis | 22 ± 2$^\dagger$ (6) |

Number of mice is shown in parentheses.
* The value in the fetal liver is expressed as number per mm$^2$, and that of the testis as number per cross-section of a seminiferous tubule.
$^\dagger$ $P > 0.1$ when compared with the value of +/+ mice by *t* test.

Since the 145-kd c-*kit* protein is considered to be the functional form,[20,21,47] this may explain the normal development of germ cells in the testis of *mi/mi* mice and the poor response of *mi/mi* CMCs to SCF.[8]

An appreciable amount of mRNA of *mi* factor is detectable in the MC-6 murine mast cell line and the Melan-C murine melanocyte cell line.[15] The mRNA expression of *mi* factor is apparently detectable in the heart, uterus, and lung of mice, but barely detectable in the brain, liver, spleen, kidney, or testis.[15,16] This expression pattern of *mi* factor is consistent with the present results. The c-*kit* expression appeared to be regulated by *mi* factor in mast cells. Since *mi* factor is not expressed in the spleen, which is an erythropoietic organ even in adult mice, the c-*kit* expression may not be regulated by *mi* factor in erythropoietic cells. In fact, the number of c-*kit* protein$^+$ cells in the fetal liver (mostly erythropoietic cells) was comparable between *mi/mi* and control +/+ embryos. The c-*kit* expression appeared to be regulated by *mi* factor in melanocytes, but that of DRG neurons did not. Although the expression of *mi* factor in DRGs has not been reported, *mi* factor is not significantly expressed in the brain. Probably *mi* factor may not be produced

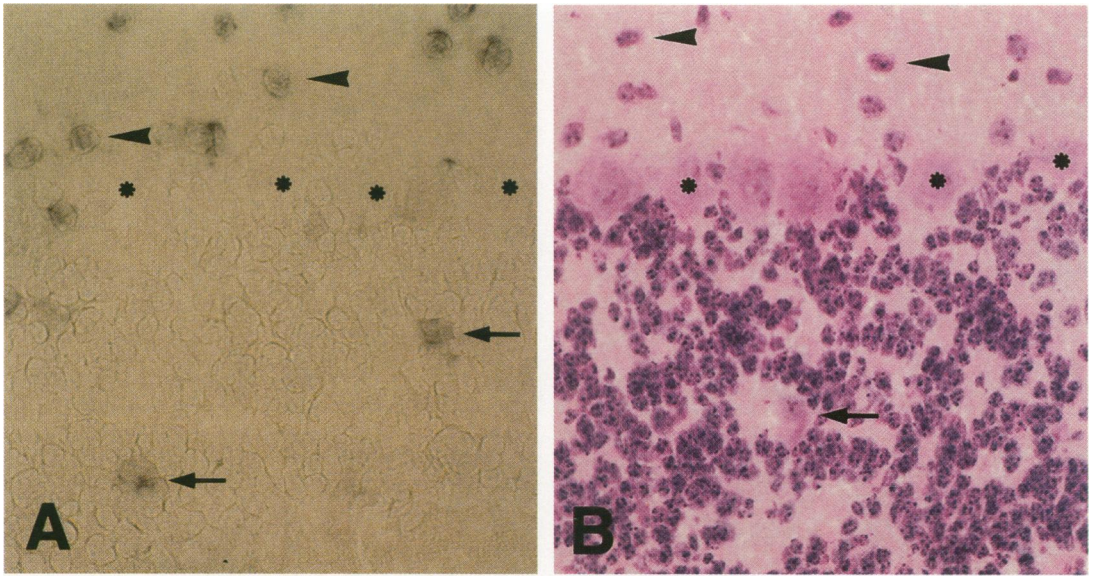


**Figure 4.** *Neurons expressing* c-kit *mRNA in the cerebellum of 20-day-old* mi/mi *mice. Basket cells in the molecular layer* (**arrowheads**) *and Golgi cells in the granular layer* (**arrows**) *express* c-kit *mRNA.* Asterisks *indicate Purkinje cells. The location of neurons can be recognized by comparing* **A** *with* **B**. **A:** *demonstration of* c-kit *mRNA*$^+$ *cells by* in situ *hybridization.* **B:** *Section stained with H & E from the same cerebellum shown in* **A**. ×760.

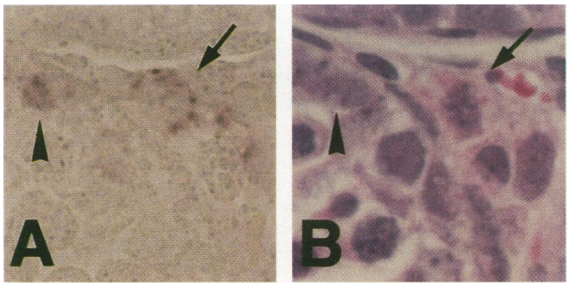


**Figure 5.** *Section of testis from a 20-day-old* mi/mi *mouse.* **A:** *demonstration of* c-kit *mRNA*$^+$ *cells by* in situ *hybridization.* **B:** *Adjacent section of* **A** *stained with H & E* (×760). **Arrowheads** *in* **A** *and* **B** *indicate the same spermatogonium, and the* **arrows** *in* **A** *and* **B** *the same Leydig cell.*

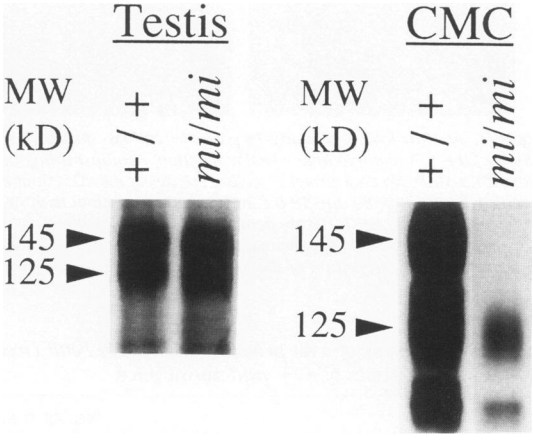


**Figure 6.** *Normal production of* c-kit *protein by testicular cells of 20-day-old* mi/mi *mice. As a control, poor production of* c-kit *protein by* mi/mi *CMCs is also shown. Both 145-kd and 125-kd* c-kit *proteins are observed in the lysate of* mi/mi *testicular cells, but only a faint band of 125-kd* c-kit *protein was detectable in the lysate of* mi/mi *CMCs.*

in DRG neurons, and therefore the c-*kit* expression does not appear to be regulated by *mi* factor in DRG neurons. The *mi* factor is not significantly expressed in the testis. This is consistent with the present result that c-*kit* is normally expressed by both germ cells and Leydig cells in the testis of *mi/mi* mice.

In the case of mast cells, two independent growth factor/receptor systems are available for development (ie, SCF/c-*kit* receptor system and interleukin-3 (IL-3)/IL-3 receptor system).[43] Therefore, CMCs of *mi/mi* mouse origin can be obtained easily in the culture containing IL-3 in spite of the poor expression of c-*kit* receptor by *mi/mi* CMCs.[7,8] If melanocytes can be obtained in the culture containing growth factor(s) other than SCF, experiments may be possible using melanocytes derived from the neural crest of *mi/mi* embryos. Although we speculate that only a small amount of 125-kd c-*kit* protein may be synthesized by *mi/mi* cultured melanocytes as in the case of *mi/mi* CMCs, this remains to be clarified.

Taken together, the present result suggested that the regulation of the c-*kit* transcription by the *mi* factor was dependent on cell types. The *mi* factor appeared to be indispensable for transcription of the c-*kit* gene in mast cells and melanocytes but not in erythroid precursors, testicular germ cells, Leydig cells, DRG neurons, and basket and Golgi cells of the cerebellum. Mast cells and erythroid cells are derived from CFU-S, and melanocytes and DRG neurons from the neural crest. Therefore, the necessity of the *mi* factor

in each cell type did not appear to be determined in the early stage of differentiation such as CFU-S and neural crest cells, but in the more differentiated stage, ie, after the commitment to the mast cell or melanocyte lineage. Mice of *mi/mi* genotype appear to be a useful model to analyze the function of transcription factors in the whole-animal level.

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