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Ectopic expression of a c-*kit*^{W42} minigene in transgenic mice: recapitulation of *W* phenotypes and evidence for c-*kit* function in melanoblast progenitors

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The proto-oncogene c-*kit* encodes a transmembrane tyrosine kinase receptor that is allelic with the murine *white-spotting* locus (*W*). *W* mutations affect melanogenesis, gametogenesis, and hematopoiesis during development and adult life, and they result from the partial or complete loss of c-*kit* function. The W^{42} allele is a *W* mutation with severe effects in both the homozygous and the heterozygous states. Previous analysis of the W^{42} allele identified a missense mutation in an essential amino acid of the c-*kit*^{W42} kinase domain that abolishes the in vitro kinase activity of the c-*kit*^{W42} protein but does not affect its normal expression. These results suggested that the c-*kit*^{W42} allele was a dominant negative mutation within the context of c-*kit*-mediated signal transduction. To further explore the dominant negative characteristics of the W^{42} mutation, we have generated transgenic mice in which ectopic expression is driven by the human β-actin promotor (hAP). Two mouse lines carrying the hAP–c-*kit*^{W42} transgene show an effect on pigmentation and the number of tissue mast cells. The patchy coat color pattern of the line 695 mice may reflect variable expression of the transgene in melanoblast progenitors and their descendents and, consequently, is indicative of a function for c-*kit* in early melanoblasts. Germ cell development and erythropoiesis, however, do not appear to be affected by the transgene. Mice expressing the c-*kit*^{W42} transgene therefore recapitulate some of the phenotypes of mice with *W* mutations. These results are therefore in agreement with the molecular basis of the W^{42} mutation and the dominant-negative characteristics of the c-*kit*^{W42} protein product.

[*Key Words*: c-*kit*; dominant *W* mutation; transgenic mice; ectopic expression; melanoblast progenitors]

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The proto-oncogene c-*kit* is the normal cellular counterpart of the viral oncogene v-*kit* of the HZ4–feline sarcoma virus (Besmer et al. 1986). c-*kit* encodes a transmembrane tyrosine protein kinase that is structurally similar to the platelet-derived growth factor receptors PDGFR-A and PDGFR-B and the colony-stimulating factor-1 receptor (CSF-1R) (Yarden et al. 1987; Qiu et al. 1988). The unique features of c-*kit*, CSF-1R, PDGFR-A, and PDGFR-B are an extracellular domain with characteristics of the immunoglobulin super family and a split kinase. The proto-oncogene c-*kit* is allelic with the *white spotting* locus (*W*) on mouse chromosome 5 (Chabot et al. 1988; Geissler et al. 1988; Nocka et al. 1989). Mutations at the *W* locus are cell autonomous and affect various aspects of melanogenesis, gametogenesis, and hematopoiesis during embryogenesis and in the adult animal (Russell 1979; Silvers 1979). The ligand of the c-*kit* receptor KL has been identified and characterized recently and shown to be allelic with the murine *steel* (*Sl*) locus (Nocka et al. 1990c; Williams et al. 1990; Zsebo et al. 1990). In agreement with the ligand receptor relationship between KL and c-*kit*, *Sl* mutations affect the same cellular targets as *W* mutations; however, in contrast to *W* mutations, *Sl* mutations are not cell autonomous and they affect the microenvironment of the c-*kit* receptor.

During normal embryonic development in mice, melanocyte progenitor cells migrate from the neural crest to the periphery (Rawles 1947; Mayer 1973a). Subsequently, they move from the dermis into the epidermis and then become incorporated into developing hair follicles; after birth, amelanotic melanoblasts differentiate into mature melanin-producing melanocytes (Mayer 1973; Silvers 1979; LeDouarin 1982). *W* mutations are thought to affect early cells as well as mature cells during melanogenesis (Russell 1949; Mayer and Green 1968). In support

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of a function of c-*kit* in mature melanocytes, first, the pigmentation pattern of $Sl/Sl^{d}-+/+$ mouse aggregation chimeras indicates a function for the KL in the skin, for example, in the dermis and in the hair follicle; second, mice heterozygous for the W^{v} allele have pigment granules that are reduced both in number and size and, furthermore, melanocytes have been shown to express c-*kit* (Russell 1949; Mayer 1973b; Nakayama et al. 1988a; Nocka et al. 1989). An effect of *W* mutations on early development has been inferred from the spotting phenotype seen in *W*/+ heterozygous mice. The precise stages at which c-*kit* functions during the early phase of melanogenesis, however, are not well defined.

Primordial germ cells migrate from the yolk sac splanchnopleure to the germinal ridges, and, subsequently, oogenesis and spermatogenesis proceed according to well-defined developmental programs. Severe *W* and *Sl* mutations affect the early phase of germ cell development (Bennett 1956; Mintz and Russell 1957; McCoshen and McCallion 1975), and mutations with mild phenotypic effects are deficient in aspects of postnatal germ cell development (Coulombre and Russell 1954; Geissler et al. 1981; Kuroda et al. 1988; Nakayama et al. 1988b). In agreement with effects of *W* and *Sl* mutations on early and more mature germ cells, c-*kit* expression has been demonstrated in primordial germ cells, proliferating fetal gonia, during postnatal oocyte development, and during spermatogenesis (Manova et al. 1990; Orr-Urtreger et al. 1990; Sorrentino et al. 1991; Manova and Bachvarova 1991).

During development prior to day 10, the blood islands in the embryonic yolk sac are the major site of hematopoiesis; subsequently, hematopoietic stem cells migrate to the fetal liver and to the sites of neonatal and adult hematopoiesis (Moore and Metcalf 1970). The hematopoietic defects in *W* mutant animals are first detected during yolk sac hematopoiesis and then persist throughout development and in the adult animal (Russell et al. 1968). In hematopoiesis *W* mutations typically affect pluripotential precursors as well as distinctive cell populations in the erythroid cell lineage and tissue mast cells; and mice homozygous for severe *W* mutations die perinatally, presumably of macrocytic anemia (Kitamura et al. 1978; Russell 1979).

A large number of independent mutations are known at the *W* locus. The different alleles vary in their effect on the different cell lineages in the heterozygous and the homozygous states (Silvers 1979; Geissler et al. 1981; Lyon and Searle 1989). The molecular bases of several of these mutations have been determined and were found to result from the partial or complete loss of c-*kit* function (Nocka et al. 1989, 1990a; Reith et al. 1990; Tan et al. 1990). The W^{42} allele is a severe dominant *W* mutation with parallel effects on pigmentation, gametogenesis, and hematopoiesis (Geissler et al. 1981). Homozygous W^{42}/W^{42} mice die perinatally presumably from severe macrocytic anemia. Mice heterozygous for the W^{42} allele are completely white, have gonads that are reduced in size, and suffer from macrocytic anemia. The phenotype of heterozygous $W^{42}/+$ mice is more severe than that of mice heterozygous for a *W* null allele; hence, the W^{42} mutation has dominant characteristics. The W^{42} allele results from a missense mutation that replaces aspartic acid 790 of the c-*kit* protein product with asparagine (Tan et al. 1990). Aspartic acid 790 is a conserved residue in all protein kinases; consequently, the W^{42} c-*kit* protein product lacks tyrosine kinase activity, but its size and cell surface expression are not affected by the mutation. Ligand-induced signal transduction by protein tyrosine kinase receptors involves the formation of receptor dimers/oligomers as intermediates (Yarden and Schlessinger 1987; Heldin et al. 1989). Therefore, the presence of mutant W^{42} c-*kit* protein in receptor heterodimers presumably interferes with c-*kit* ligand-induced signal transduction, reducing the number of functional c-*kit* receptors on the cell surface. The W^{42} allele consequently has the characteristics of a dominant-negative mutation.

To further investigate the dominant-negative characteristics of the c-kit^{W42} gene product in vivo and to better understand its effects on the various cell lineages, we sought to construct transgenic mice that express the c-kit^{W42} protein ectopically. The predicted outcomes of such an experiment are the following: (1) The expression of the c-kit^{W42} transgene in cells that also express the endogenous c-*kit* gene should result in phenotypic effects similar to those seen in $W^{42}/+$ and W^{42}/W^{42} mice; (2) the expression of the c-kit^{W42} transgene in cells that also express the c-*kit* ligand might effect the removal of the ligand and therefore perturb normal c-*kit* function; (3) expression of the c-kit^{W42} transgene in other cells should be of no consequence, because the transgene is functionally inactive. We therefore constructed transgenic mice in which the c-kit^{W42} cDNA was expressed under the control of the constitutive human β-actin promoter (hAP). Two mouse lines containing the c-kit^{W42} transgene were obtained and characterized. The hAP–c-kit^{W42} mice display varying degrees of white-spotting and have a reduced number of mast cells in the skin. These results indicate that the hAP–c-kit^{W42} transgene in these mice causes the recapitulation of some *W* phenotypes and provide evidence for c-*kit* function in early melanogenesis.

Results

Transgene construct

To achieve ectopic expression of the c-kit^{W42} cDNA in transgenic mice, a minigene cassette was made, consisting of the c-kit^{W42} cDNA, the hAP, and the SV40 polyadenylation site. An intron from the human β-actin gene was included in this minigene, because it has been shown that intron sequences may increase the transcriptional efficiency of cDNA constructs in transgenic mice, possibly due to the presence of transcriptional control elements (Brinster et al. 1988; Palmiter et al. 1991). hAP construct used in this study consists of 3 kb of 5′-flanking sequence from the human β-actin gene, exon 1 containing 5′-untranslated sequence, the first intron with its

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3′-splice site and an SV40 polyadenylation site. It has been shown previously to be an excellent vehicle for high-level transgene expression (Gunning et al. 1987; I.R. Lemischka, pers. comm.). The c-*kit*^{W42} cDNA was inserted into the *Sal*I site of the pHβAPr-1 expression vector to generate the hAP–c-*kit*^{W42} transgene (Fig. 1A). To verify the c-*kit*^{W42} protein products specified by the hAP construct, NIH-3T3 cells were cotransfected with the hAP–c-*kit*^{W42} construct and with pSV2neo, and stable transfectants were obtained upon selection with G418. Cells in individual colonies were expanded and examined by immunoprecipitation analysis of [^{35}S]methionine-labeled cell extracts, and immune complex kinase reactions were performed to analyze the c-*kit*-associated kinase activity. SDS-PAGE of immunoprecipitates shows the expression of the expected 160- and 130-kD c-*kit*^{W42} protein products from the hAP–c-*kit*^{W42} construct (Fig. 1B), and analysis of products of immune complex kinase reactions indicate that the c-*kit*^{W42} protein products lack autophosphorylation activity (not shown).

Generation of transgenic mice

To generate transgenic mice the 8.7-kb *Cla*I fragment, which comprises the hAP–c-*kit*^{W42} minigene (Fig. 1A), was injected into the pronuclei of fertilized (C57BL/6J × CBA/J) F_1 eggs. Microinjected eggs were then transferred into the oviducts of pseudopregnant CD1 females. Offspring were tested for the presence of the transgene by analyzing tail DNA. Tail DNA was digested with the restriction enzyme *Bam*HI, which generates an internal 3.6-kb transgene fragment, and the DNA blots were analyzed by hybridization with a c-*kit* probe. Of the 35 offspring, 2 male and 2 female mice were found to carry the transgene. One female founder animal (697) had a white coat except for pigmented areas around the ears and at the end of the trunk. Upon breeding, this animal produced only one litter of three pups, none of which survived. Two founder animals, 485 and 676, had normal coat pigmentation, whereas the fourth, 695, had normal coat pigmentation except for a white spot on the forehead. Transgenic lines were established from the 485 and 695 founder animals by mating with (C57BL/6 × CBA/J) F_1 mice. Only 25% of the offspring produced by the 695 founder male contained the transgene, raising the possibility that the germ-line tissue in this mouse is mosaic (Fig. 2).

Coat color phenotypes of line 485 and 695 mice

Although there was no visible effect on pigmentation in founder female line 485 (data not shown), progeny produced by mating this mouse with a B6CB F_1 male displayed white-spotted areas in different parts of the body (Fig. 3A). Typically, these animals have a white spot on the forehead and on the ventral side. They most often had pigmentless feet and a banded tail and, less often, had other white areas on the body. However, the pigment pattern among littermates and their offspring varied and, therefore, did not appear to be a genetically transmitted characteristic (Fig. 3A). Founder male 695 had a diamond-shaped white spot on the forehead (data not shown). The offspring of the 695 mouse have a unique spotting pattern on their trunk (Fig. 3B). Pigmented areas, "splotches," may extend from the dorsal midline toward the ventral side, indicating that the two sides of the animal are formed independently. Feet and tail most often lack pigment, although a banded tail pattern is seen as well. The coat color pattern of the 695 mice again was variable and thus was was not a genetically stable transmitted trait. All 485 and 695 mice have black eyes, as do mice homozygous for various *W* alleles. In 485 and 695 offspring observed over an extended period of breeding, the pigmentation pattern varied considerably between litters of the same line indicating variable penetrance. The variable penetrance could be the result of chromosomal position effects or differences in genetic background among the offspring. To determine

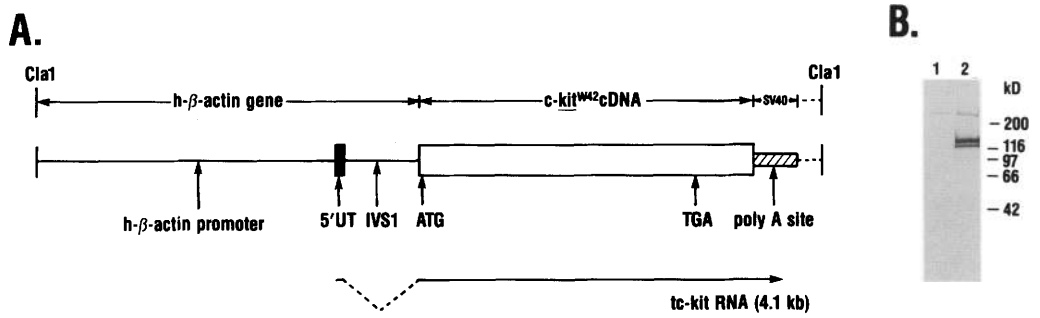

Figure 1. hAP–c-*kit*^{W42} construct and protein products. (*A*) Schematic of the 8.7-kb hAP–c-*kit*^{W42} minigene. The human β-actin gene fragment consisting of 3 kb of 5′-flanking sequence, 0.078 kb of 5′-untranslated region (5′UT), 0.832 kb of intervening sequence 1 (IVS 1), as well as the 3.7-kb c-*kit*^{W42} cDNA and the 0.5-kb SV40 fragment containing the polyadenylation signal are indicated. The spliced hybrid hAP–c-*kit*^{W42} RNA transcript is shown below. (*B*) Immunoprecipitation analysis of the c-*kit*^{W42} protein products in NIH-3T3 cells containing the hAP–c-*kit*^{W42} minigene construct (derived by cotransfection with pSV2neo and selection with G418). Cells were metabolically labeled with [^{35}S]methionine (50 μCi/ml), and cell lysates were immunoprecipitated with anti-*kit* antiserum and analyzed by 7.5% SDS-PAGE. (Lane *1*) Control cells transfected with pSV2neo; (lane *2*) cells transfected with hAP–c-*kit*^{W42} and pSV2neo plasmids. The migration of size marker proteins is indicated in kilodaltons (kD).

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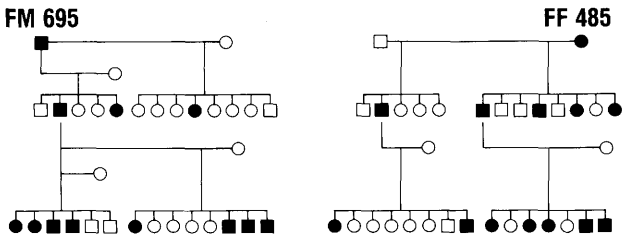

Figure 2. Pedigrees of hAP–c-*kit*^{W42} transgenic mice. The founder animals 485 and 695 were mated to (C57BL/6 × CBA/J) F_1 males or females as indicated. (□) Male mice; (○) female mice. Male (■) mice and female (●) mice are identified that contain the transgene. Four litters in the pedigrees of mice derived from the 695 founder male and the 485 founder female are shown. (FM,FF) Founder male and founder female, respectively.

whether this variable penetrance stems from the inconstant mixture of CBA and C57Bl6 genes present in the transgenic offspring, both the 485 and 695 lines are currently being established on a C57Bl/6 background. Taken together, these results demonstrate an effect of the hAP–c-*kit*^{W42} transgene on pigment formation which in many respects, is reminiscent of the effects of *W* mutations on pigmentation; the splotchy appearance of the pigment patterns in these mice, however, is unique.

Characteristics of hematopoietic parameters in line 485 and 695 mice

The hematopoietic system in mice with mutations at the *W* locus is affected in several ways. Effects are seen in the stem cell compartment, in the erythroid cell lineage, and in mast cells. To evaluate whether transgene expression in line 485 and 695 mice affects the erythroid cell lineage, erythrocyte numbers and hematocrit values were determined. The results shown in Table 1 indicate that there is no significant difference between samples from transgenic mice and corresponding nontransgenic littermates.

Mice with *W* mutations typically lack tissue mast cells; and in vitro, bone marrow mast cells from *W* mutant mice do not proliferate in response to KL. To evaluate whether transgene expression affects mast cells in lines 485 and 695, we determined the number of mast cells in skin sections from these mice. Our results (shown in Table 1) revealed a significantly reduced number of mast cells in the line 485 and 695 mice compared to that in the nontransgene control mice. Line 695 mice

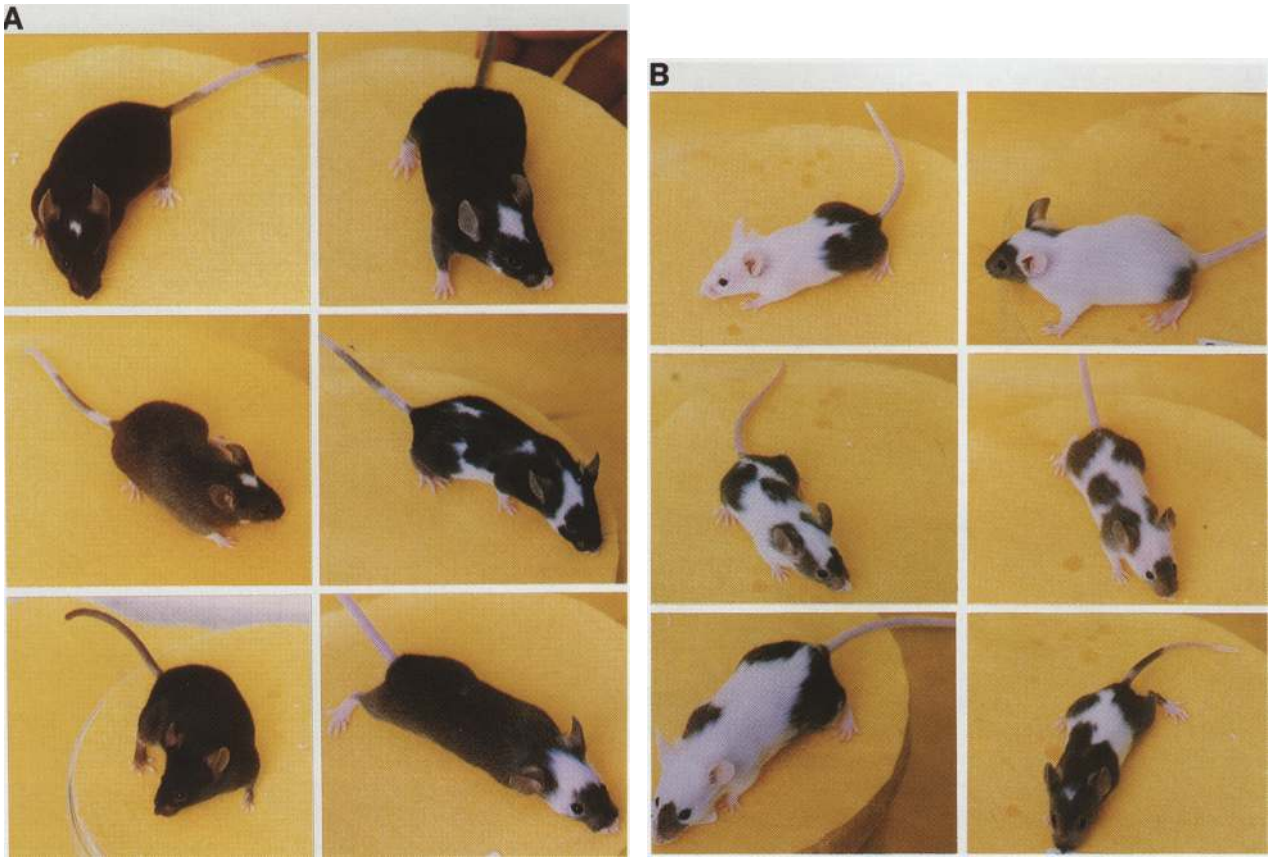

Figure 3. Coat color phenotypes of line 485 (*A*) and line 695 (*B*) mice.

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Table 1. *Influence of the* c-kit^{w42} *transgene on mast cell numbers and blood tissue of mice*

Strain	Area of skin	Number of mast cells (per cm of skin)	Number of erythrocytes ($\times 10^6$/mm^3)	Hematocrit (%)
Line 485	pigmented	40.7 ± 6.7	10.0 ± 0.2	50.3 ± 0.9
Line 695	pigmented	120.0 ± 9.3	10.0 ± 0.2	50.3 ± 0.9
	nonpigmented	139.0 ± 14.6		
Control	pigmented	364.7 ± 28.5	10.0 ± 0.2	52.2 ± 0.2
$W^{42}/+$	nonpigmented	10.3 ± 0.63	6.5 ± 0.12	45 ± 0.05
W/W^v	nonpigmented	0	7.1 ± 0.05	44.5 ± 0.5

Mice were 3–4 months old at the time of study.
The number of mast cells was counted in more than two well-separated sections in each skin sample and from six mice in each group of transgenics; nontransgenic littermates served as controls. Numbers are mean values.

contained 31% of the number of mast cells compared to their nontransgenic littermates, and there was no significant difference in the number of mast cells between the pigmented and nonpigmented areas of the skin in this line. In line 485 mice the number of mast cells in the skin was only 8% of that in the nontransgenic littermates (Table 1), although the effect of the transgene on skin pigmentation in these mice was less prominent than in the line 695 mice (Fig. 3A). These results suggest that transgene expression in 485 and 695 mice affects mast cell development to differing degrees; however, there appears to be no detectable effect of transgene expression on erythropoiesis in these two lines.

Characteristics of gonads in line 485 and 695 mice

Gametogenesis in mice with *W* mutations is affected in several ways, resulting in different degrees of infertility. Severe *W* and *Sl* mutations affect the early phase of germ cell development (Bennett 1956; Mintz and Russell 1957; McCoshen and McCallion 1975), and mutations with mild phenotypic effects are deficient in aspects of postnatal germ cell development (Coulombre and Russell 1954; Geissler et al. 1981; Kuroda et al. 1988; Nakayama et al. 1988b). To determine whether there is an effect of the transgene on gametogenesis we have examined the gonads of line 485 and 695 mice. Both the ovaries and the testes in these mice were found to be unaffected by the transgene, that is, the number and size distribution of follicles at different stages of postnatal development do not deviate from controls and spermatogonial development similarly was not affected; in agreement with these findings, the litter size of the transgenic mice was normal.

Expression of transgene RNA products in tissues of line 485 and 695 mice

The hAP–c-*kit*^{W42} minigene was designed to achieve ectopic expression of the transgene in transgenic mice. To evaluate the performance of this minigene, the expression of transgene RNA transcripts in different tissues was determined by RNA blot analysis by using a c-*kit* hybridization probe. The 5.5-kb endogenous c-*kit* RNA transcript was detectable in the brain, lung, thymus, spleen, kidney, ovary, testis, skeletal muscle, skin, and bone marrow (Fig. 4 and data not shown) as described previously, with relatively high levels in brain, lung, kid-

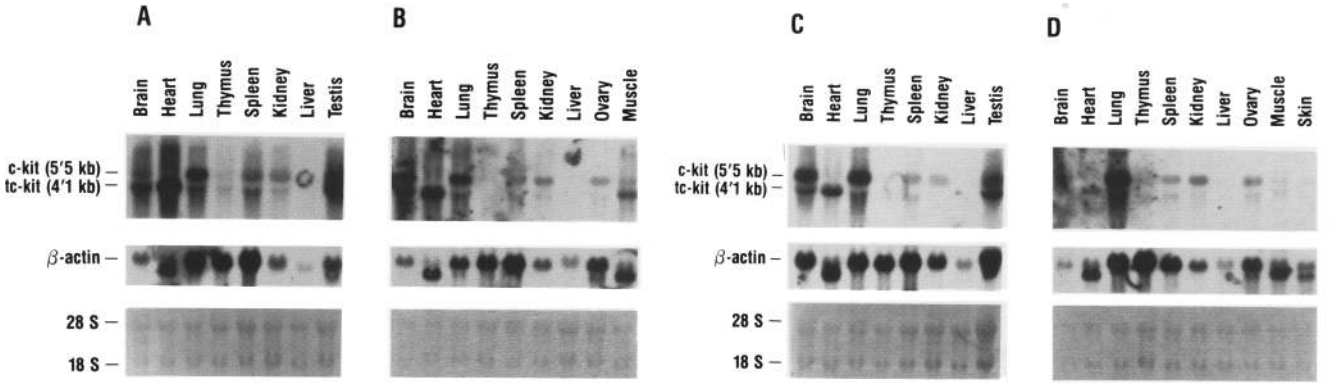

Figure 4. RNA blot analysis of c-*kit* and tc-*kit* RNA transcripts in tissues from line 485 and 695 mice. Total RNA was isolated from different tissues of 10-week-old mice. Ten micrograms of total cell RNA was electrophoretically separated in an agarose gel, blotted, and hybridized with a ^{32}P-labeled c-*kit* cDNA probe. (*Top*) The 5.5-kb endogenous c-*kit* RNA (c-*kit*) and the 4.1-kb c-*kit* transgenic RNA (tc-*kit*) are indicated. For comparison, hybridization with an actin probe is shown in the *center*. To demonstrate equal loading, methylene blue staining of the blot is shown at the *bottom*. The migration of 28S and 18S RNA is indicated. (*A*) Male line 485 mouse; (*B*) female line 485 mouse; (*C*) male 695 line mouse; (*D*) female line 695 mouse.

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ney, testis, and ovary and somewhat lower levels in skin, muscle, and thymus. The 4.1-kb hAP–c-*kit*^{W42} transcript was expressed at high levels in brain, heart, testis, and muscle and at lower levels in lung, thymus, spleen, kidney, and skin (Fig. 4). In brain, testis, muscle, and skin transgene expression is higher than that of the endogenous c-*kit* gene. In the other tissues the reverse applies. Transgene expression was essentially the same in line 485 and 695 mice and among males and females (Fig. 4,A–D). To compare RNA steady-state levels of the minigene with those of the endogenous β-actin gene the RNA blots were hybridized with an actin probe. Our results indicate that in the tissues tested, the transgene is expressed, but at different levels, and that transgene expression does not parallel β-actin expression. As a cautionary note, no conclusions in regard to transgene expression on a per cell basis can be drawn from these blotting experiments. Taken together, however, these results demonstrate variable expression of the hAP–c-*kit* transgene in different tissues at higher or lower levels than the endogenous c-*kit* gene.

Discusssion

Previous work indicated allelism between the c-*kit* gene and the murine *W* locus. The characterization of a number of dominant *W* alleles (W^{42}, W^{37}, W^{v}, W^{57}, and W^{41}), which display a strong heterozygous mutant phenotype, has shown that they result from missense mutations that affect the kinase activity but not the synthesis and cell surface expression of the mutant c-*kit* proteins. In contrast, severe *W* alleles, which give rise to weak heterozygous phenotypes such as W^{19H} and *W*, have been shown to be c-*kit* null mutations. The dominant nature of *W* mutations, such as W^{42}, suggests that the mutant protein inhibits normal *kit* ligand-induced signal transduction. Presumably, this inhibition results from the formation of receptor heterodimers (or oligomers), suggesting that receptor dimers/oligomers are an essential intermediate in KL-induced receptor activation. To further explore the dominant-negative characteristics of the W^{42} allele, we have constructed transgenic mice that express the c-*kit*^{W42} gene products ectopically. The conclusions from these experiments are twofold: (1) ectopic expression of the c-*kit*^{W42} cDNA in transgenic mice, achieved by using the human actin promotor to direct the synthesis of the c-*kit*^{W42} gene products, results in the recapitulation of some *W* phenotypes, for example, pigmentation and mast cell deficiency; (2) c-*kit* must function in early melanoblast progenitors.

During normal development from day 9 to day 12, melanoblasts migrate within the dermal mesoderm from the neural crest to the periphery and then enter the epidermal ectoderm and colonize developing hair follicles. By using tetraparental mice, Mintz noticed the formation of patterns of transverse bands of two colors on the head, trunk, and tail (Mintz 1967, 1971). Along the body axis there was a discontinuity, suggesting that the pattern of stripes on the two sides was formed independently. Therefore, Mintz proposed that the pigment system is derived from 17 pairs of melanoblast stem cells that proliferate and migrate laterally and ventrally, from the neural crest, establishing a pattern of transverse bands. Recently, in utero transplantation of neural crest cells was shown to facilitate the formation of neural crest chimeras with pigmentation patterns in agreement with the Mintz hypothesis (Huszar et al. 1991). The coat color patterns in the tetraparental mouse chimeras of Mintz and those seen in the line 695 mice are alike. To display a dominant-negative effect in melanogenesis, the transgene must be expressed in melanoblasts/melanocytes in some or all developmental stages at which c-*kit* exerts an essential function. Thus, the pigmentation pattern seen in the transgenic mice may represent melanoblast clones revealed by the expression of the c-*kit*^{W42} transgene in some melanoblast founder cells and their progeny and not in others, or in the descendants of melanoblast founder cells. In agreement with this notion, c-*kit* expression has been documented in cells along the presumptive path of melanoblast migration at 10–11 days of embryonic development (Manova and Bachvarova 1991). In the chimeric mice of Mintz, the coat color patterns consist of lateral stripes; in the transgenic mice, the pigmented areas have a splotchy appearance. In *W* mutant mice nonpigmented areas of the skin are either devoid of melanocytes or they contain defective melanocytes. Therefore, viable melanocytes from pigmented skin regions may migrate into adjacent nonpigmented regions, thus extending the pigmented stripes into splotches (Huszar et al. 1991). *W*/*W*-+/+ aggregation chimeras phenotypically resemble *W*/+ mice and are not striped but have a ventral spot (Mintz 1971). Mintz argued that as a result of lack of cell proliferation or cell death of the migrating *W*/*W* melanoblasts, progenitors from a neighboring +/+ melanoblast clone may invade the territories of nonviable clones but that the total number of +/+ cells is decreased compared with that of a +/+ animal, accounting for the ventral spot. In line 695 mice a banded pattern can be discerned because the number of pigmented clones is relatively small as seen from the lack of pigmentation of the tails. In line 485 mice a banded pattern is less apparent because more clones are pigmented. The observation of a banded pigmentation pattern in line 695 mice therefore indicates that c-*kit* function is essential for the proliferation and survival of melanoblasts in early development.

The variability of the coat color phenotype appears to be the result of clonal variation of the expression of the transgene. In transgenic mice produced by pronuclear injection, the site of chromosomal insertion of the transgene is presumed to be random. Although the copy number of the transgene varies greatly from one integration site to another, there is no general relationship between the copy number and the level of expression (Hammer et al. 1987). However, it is very well established that expression of transgenes is influenced by position effects imposed by *cis*-acting factors at the integration sites and by strain-specific *trans*-acting factors that may induce epigenetic modification of these loci (Lacy et al. 1983; Allen et al. 1988, 1990; Kothary et al. 1988; Engler et al.

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1991). Thus, position effects and/or differences in genetic background may determine the variations in penetrance and expressivity observed in line 695 and 485 mice. Taken together, our results provide evidence for a function of c-*kit* in the early developmental stages of pigment formation.

In the hematopoietic system, *W* mutations typically affect pluripotential precursors, distinctive cell populations in the erythroid cell lineage, and mast cells. The analysis of the hematopoietic parameters in line 485 and 695 mice indicates, on one hand, no effect of transgene expression on erythropoiesis but, on the other hand, shows a reduction of mast cells in the skin of the line 485 and 695 mice compared with normal controls. These results suggest that the transgene is expressed in mast cells and affects c-*kit* function in this cell type. In line 485 mice the number of mast cells is only 8% of that present in normal littermates, whereas in line 695 mice the number of mast cells is 30–40% of that in control animals. This result is in contrast to the pigmentary aspects of the line 485 and line 695 mice. On one hand, these results may indicate different levels of transgene expression in mast cells and melanoblasts of the two lines, and on the other hand, they may reflect the different microenvironments of connective tissue mast cells and melanocytes in the skin; whereas mast cells are found only in the dermis, melanocytes are found in the dermis and in the epidermis (hair follicles) (Nakayama et al. 1988a). Experiments are currently in progress in which we will investigate the in vitro properties of bone marrow-derived mast cells isolated from line 485 and 695 mice. The lack of an effect of the transgene on erythropoiesis is also of interest. This result may indicate that the level of expression of the transgene in erythroid progenitors is not sufficient to inhibit c-*kit* function in these cells.

In the experiment described here we expressed the c-*kit*^{W42} gene products ectopically to evaluate c-*kit* function in a broad range of cell types in vivo. The success of this approach argues that by using cell lineage-specific promoters it should be possible to express the c-*kit*^{W42} gene products and inhibit c-*kit* function in specific cell types. A function in cell differentiation and development is not known for many mammalian kinase receptors as a result of the scarcity of germ-line mutations. Null mutations in these receptor genes, presumably like *W* mutations, may be recessive lethal mutations. The cell type-restricted or ectopic expression in transgenic or chimeric mice of newly manufactured dominant-negative mutations, like c-*kit*^{W42}, may therefore help define the function of tyrosine kinase receptors in development and differentiation.

Materials and methods

Construction of c-kit^{W42} transgene

A 3713-bp *Eco*RI–*Hin*dIII fragment of the c-*kit* cDNA, which contains the complete c-*kit*-coding sequence (2956 bp), 28 bp of the 5′-untranslated region and 757 bp of the 3′-untranslated region, was cloned into pGEM-7Zf (+) (Promega). The 3′ *Hin*dIII site was modified by the addition of *Xho*I linkers. The single mutation present at nucleotide position 2396 in c-*kit* cDNA isolated from mast cells of W^{42} homozygous mice was introduced into the c-*kit* cDNA by exchanging the *Sty*I–*Bgl*II fragment (nucleotides 2352–2802 of the c-*kit*-cDNA) with the corresponding fragment isolated from the c-*kit*^{W42}-cDNA. Preservation of the rest of the sequence was confirmed by DNA sequencing. The resulting c-*kit*^{W42}-cDNA was digested with *Xho*I and cloned into the *Sal*I site of the expression vector pHβAPr.2 (Gunning et al. 1987). Clones containing the coding sequence of the c-*kit*-cDNA in the proper orientation with respect to the hAP were selected by digesting the DNA with *Bam*HI. All DNA manipulations were carried out according to established procedures (Sambrook et al. 1989).

Generation of transgenic mice

Plasmid DNA containing the human hAPc–*kit*^{W42}-cDNA minigene was digested with *Cla*I, and the 8.7-kbp transgene fragment was purified by agarose gel electrophoresis. Approximately 100 μg of transgene DNA was dissolved in 13 ml of TE buffer [10 mM Tris-HCl (pH 7.5)/1 mM EDTA (pH 8.0)] containing 1.68 gram of CsCl/ml and centrifuged at 37,000 rpm in a Ti75 rotor (Beckman) for 48 hr at 20°C. Fractions (0.5 ml) were collected from the gradient, 3-μl aliquots of each fraction were analyzed by agarose gel electrophoresis, and the fractions containing the DNA fragment were then dialyzed against the injection buffer [5 mM Tris-HCl (pH 7.4)/5 mM NaCl/0.1 mM EDTA]. The DNA concentration was adjusted to 200–400 molecules/picoliter, and pronuclear microinjections were performed by following standard techniques (Hogan et al. 1986). Specifically C57BL/6J or (C57BL/6J × CBA/J)F_1 (B6CB F_1) female mice were superovulated and mated with B6CB F_1 males. The fertilized eggs were recovered, and DNA was microinjected into pronuclei. Transgenic lines were propagated by mating with (C57BL/6 × CBA/J) F_1 mice.

DNA blot analysis

Mouse tail-tip DNA was prepared by removing ~1 cm of tail and incubation in 0.5 ml of tail-tip buffer [50 mM Tris-HCl (pH 8.0)/0.1 M EDTA/0.5% SDS/1 mg/ml of proteinase K] at 55°C overnight. After the addition of 0.1 volume of 3 M sodium acetate the hydrolysate was extracted with an equal volume of phenol/chloroform (4% isoamyl alcohol) (1 : 1). The DNA in the aqueous phase was precipitated by the addition of 0.6 volume of isopropanol, and the precipitate was washed twice with 70% ethanol. Twenty micrograms of DNA was digested with *Bam*HI, and 8 μg of the digested DNA was separated by agarose gel electrophoresis, transferred to nitrocellulose following the procedure of Southern (1975), and probed with a ^{32}P-labeled c-*kit* cDNA fragment. Hybridization was carried out in 6× SSC [1× SSC = 0.15 M NaCl/0.015 M sodium citrate (pH 7.0)], 1× Denhardt's solution (0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% BSA), and 50 μg/ml of denatured salmon sperm DNA for 24 hr at 42°C. Filters were washed for 20 min in 2× SSC/1% SDS at room temperature, for 30 min each in 0.2× SSC/1% SDS and in 0.1× SSC/1% SDS at 55°C prior to autoradiography.

RNA blot analysis

RNA was prepared from tissues of adult (2-month-old) mice according to the method of Chomczynski and Sacchi (1987). Five micrograms of total RNA from each tissue was electrophoretically fractionated in 1% agarose gel containing 2.2% formaldehyde in MOPS buffer (Sambrook et al. 1989), transferred to

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nitrocellulose, and probed with a ^{32}P-labeled c-*kit* cDNA fragment. Hybridization was at 42°C for 18 hr in 6× SSC/1× Denhardt's solution/50% formamide containing 10^6cpm/ml of probe. Blots were washed twice for 15 min at room temperature in 1× SSC/0.1% SDS, twice for 15 min at 55°C in 0.2× SSC/0.1% SDS, and then subjected to autoradiography. After stripping the blot of the c-*kit* probe, the same blot was rehybridized with a β-actin probe.

Identification of skin pigmentation

Because the mice displayed extensive and/or irregular spotting patterns, it was difficult to quantify the extent of white spotting. For qualitative characterization, each mouse was observed carefully for (1) any dilution of coat color other than the agouti and the C57Bl6 phenotypes and (2) white spotting in the dorsal and ventral parts. To assess any defined patterns of white spotting, photographs of mice belonging to the same line were compared by aligning corresponding dorsal or ventral views side by side.

Determination of mast cell number in the skin of transgenic and control mice

Mice were sacrificed by cervical dislocation. Pieces of dorsal skin were removed from different parts, smoothed onto a piece of thick filter paper to keep them flat, and fixed in PBS–10% formalin (pH 7.2). Tissues were embedded in paraffin, and 5-μm sections were stained with 0.1% acidified toluidine blue (pH 3.0). In the skin sections, mast cells between the epithelium and paniculus carnosus were counted under the microscope. The number of mast cells/per centimeter length of skin was determined by dividing the mast cell number by the length of each section of the skin counted. For each sample, measurements were made in two separate histological sections and averaged to provide the sample mean. Nontransgenic littermates were used as controls.

Hematology

Blood samples were collected from tail tips into heparinized Red Tip microhematocrit capillary tubes and sedimented by centrifugation for 3 min in an Autocrit centrifuge, and hematocrits were determined with the aid of a Clay-Adams microhematocrit reader. For red blood cell (RBC) count, 10 μl of blood was diluted 10,000-fold in PBS and counted in a Coulter cell counter. Nontransgenic littermates were used as controls.

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