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Spontaneous malignant transformation of melanocytes explanted from W^f/W^f mice with a *Kit* kinase-domain mutation

(protooncogene/growth factor receptor/melanoma/programmed cell death/mosaic gene expression)

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ABSTRACT **The *W/Kit* mouse locus, affecting proliferation and survival of pigment cells, blood cells, and germ cells, is known to encode a tyrosine kinase growth factor receptor and is considered a protooncogene; yet it has not heretofore been causally implicated in any malignancies of those cells. The W^f/W^f mutant mouse coat comprises viable and inviable melanoblast clones, seen ultimately as pigmented and white transverse stripes—the latter more prominent. Judging from the pattern, all clones initially expand, and the inviable ones then undergo programmed cell death prenatally. To observe skin melanocytes of the viable clones during extended proliferation, the cells were explanted from individual young mice. An unusually large number of primary explants failed to survive—a result consistent with a growth handicap. In 3 of the 10 surviving cell lines, many cells spontaneously underwent a series of striking changes with the classic features of transformation. The two transformed lines that have been tested by grafting to immunosuppressed hosts formed undifferentiated invasive tumors compatible with malignant amelanotic melanoma. None of our 52 other melanocyte lines of the coisogenic wild-type strain and 13 other natural genotypes have become transformed under the same culture conditions. Molecular analysis of the W^f gene revealed a single change from wild-type: a point mutation affecting the catalytic region in the kinase domain of the Kit protein. The apparent growth disadvantage due to the mutation may allow selection for melanocytes mobilizing more efficient pathways, thus leading to neoplasia. Production of both viable and inviable melanoblast clones is unlikely to be due only to the kinase mutation; possibly the degree, duration, and consistency of expression of this locus may be controlled by cis elements outside the coding region.**

The *W* locus on mouse chromosome 5, long known to affect pigment cells, blood cells, and germ cells (1–3), has been found to correspond to mouse c-*kit*, now designated *Kit*, encoding a transmembrane tyrosine kinase growth factor receptor (4–6) for a ligand produced by the steel (*Sl*) locus (reviewed in ref. 7). c-*kit* is considered a protooncogene according to structural criteria, but a causal role in oncogenesis has not been demonstrated. A few observational and experimental approaches have been used to investigate its possible oncogenic function. The level of c-*kit* RNA or protein has been observed to drop markedly in melanomas as compared with melanocytes (8, 9), although this may be either a cause, a correlate, or a consequence of transformation. In contrast, a tumor derived from mouse mast cells (in which the *W* locus is normally expressed) was found to have a very high level of Kit RNA (8). The general difficulty in interpreting the significance of the level of *Kit* expression also emerges from various studies (e.g., ref. 10) documenting expression in many tissues of the mouse embryo in addition to those with known defects due to *W*-locus mutations. In one experimental approach to the question of oncogenicity, antisense oligomers were used to disrupt human c-kit function; this inhibited growth of some kinds of cultured hematopoietic cells or leukemic colonies and not others (11). In another study, a chimeric receptor molecule was constructed from the extracellular domain of the epidermal growth factor receptor and a *Kit* transmembrane and cytoplasmic portion; its activation in fibroblasts stimulated kit activity and cell proliferation and resulted in a transformed phenotype (12).

Our experiment takes advantage of a *W*-locus mutant, W^f/W^f, whose coat color phenotype is consistent with adverse effects of the mutation on melanocyte proliferation and viability. We report here that the mutation is in the *Kit* kinase domain and that cell lines established from W^f/W^f skin melanocytes have undergone spontaneous malignant transformation in a significant number of cases.

MATERIALS AND METHODS

Melanocyte Cell Lines. Skin melanocytes were explanted from mice 1–6 days old and cultured in the standard medium as described (13, 14). Flasks were split at subconfluence, and the long-term doubling times were estimated weekly. Established lines were frozen as described (15). Cell lines of the following *W* mutant genotypes (2, 16) were obtained: W^f/W^f and $W^f/+$, both on the C3H inbred strain background, with wild-type C3H as control; and $W^v/+$ and $W/+$, both on the C57BL/6 background, with wild-type C57BL/6 as control.

Assays for Transformation and Tumorigenicity. Short-term growth curves were obtained after phorbol 12-myristate 13-acetate (PMA) was omitted from the medium and also after the concentration of fetal calf serum was lowered from 20% to 2% in medium lacking PMA. The cells were seeded in 25-cm^2 flasks, fed every 3–4 days, and counted 1 day after seeding and then at intervals of 2–3 days.

Melanin content was determined after the cells were centrifuged and washed in phosphate-buffered saline. One million cells were resuspended in 100 μl of 1 M NaOH and mixed for 15 min. An absorption baseline was established with 1 M NaOH. The level of melanin was read at 475 nm with various numbers of cells, and a melanin standard was obtained as a homopolymer of dopaquinone (Sigma).

To assay for anchorage independence, exponentially growing cells were diluted to 10^3, 10^4, and 10^5 cells per 3 ml in the standard culture medium with 0.33% Seaplaque agar (FMC). The cells were plated in 6-cm Petri dishes over >5 ml of 0.5% solidified agar in the standard medium and were fed regularly with that medium containing 0.33% agar. After 2.5 wk, *P*-iodo-nitrotetrazolium violet (2 mg/ml; Sigma) was placed over the agar and hydrolyzed by living cells at 37°C to

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Abbreviation: PMA, phorbol 12-myristate 13-acetate.

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produce a purple color facilitating detection and counting of any nonmelanized colonies.

Exponentially growing cells were tested for tumorigenicity by injecting $\approx 2 \times 10^6$ cells subcutaneously into 6- to 8-wk-old nude (*nu/nu*-C57BL/6) mice. Tumors were fixed in formalin or Omnifix (Zymed Laboratories) for histology.

Photography. For phase-contrast light micrographs, cells were grown on coverslips and the PMA was removed to facilitate flattening. Transmission electron micrographs were made from cell suspensions fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in 2% agar and Epon-Araldite. Cells were fixed for scanning electron microscopy in glutaraldehyde and osmium tetroxide, dehydrated, critical-point-dried, and sputter-coated with gold.

RNA Analysis. Total RNA was extracted from exponentially growing cultured cells as described (17). Slot-blot and Northern analyses with Riboprobes for expression of Kit mRNA and Northern analysis of tyrosinase mRNA expression were carried out on Nytran (Schleicher & Schuell) as recommended by the manufacturer, with β-actin loading controls.

RESULTS

Coat Color Phenotype of W^f/W^f Mice. The W^f semidominant mutation arose spontaneously in the C3H/He inbred strain. The heterozygotes were described as having a white spot on the forehead and belly and a white tail tip; the homozygotes were described as having "extensively depigmented areas lacking any well-defined pattern" (16). However, all of the many W^f/W^f homozygotes that we have observed—descendants from the original source—display an orderly pattern consisting chiefly of pigmented and white transverse stripes on left and right sides (Fig. 1). The pattern is most clearly seen in young animals. The same basic pattern was first described in allophenic mice experimentally produced from aggregated blastomeres of two different coat color genotypes (18). The stripes were interpreted as visualizing the clonal developmental history of melanoblasts, with each stripe on each side formed by the migrating cellular progeny of a single neural crest-derived melanoblast. The locations and numbers of stripes do not correspond to any fixed structures. In the case of homozygotes like W^f/W^f and of so-called "white-spotted" heterozygous genotypes at W and other loci, the white stripes are "ghost" areas lacking pigment cells in the hairs. As in the other mice previously studied (2), histological examination of W^f/W^f skin revealed that the hair papillae in the white stripes are devoid of melanocytes (data not shown). Patterns resembling these have been experimentally obtained in allophenic mice by aggregating blastomeres of an "all-white-spotted" type, such as W/W or microphthalmia-white (Mi^{wh}/Mi^{wh}), with wild-type cells (19–21).

FIG. 1. W^f/W^f-C3H mice, 18 days old, with the characteristic transversely striped pattern produced by viable melanocyte clones (pigmented stripes) and inviable ones (white stripes). Note the marked difference in width and intensity of the viable clones in the two mice.

The pattern of W^f/W^f is in several respects indicative of a melanoblast growth defect. The presence of transverse white (null) bands along with pigmented ones is consistent with completion of melanoblast migration and proliferation, followed prenatally by death of half the clones, rather than to failure of their migration. This phenomenon was first termed "preprogrammed cell death" in earlier experiments by one of us (19, 20). Graft tests with genetic markers of skin from comparable white-striped allophenic mice to the parental strains have already shown that null areas are not attributable to any local environmental differences in the skin (refs. 19 and 22, and unpublished data). In W^f/W^f mice, the pigmented stripes are sometimes less prominent than the white ones (Fig. 1); combined with limited mixing at clonal boundaries, the viable clones appear ultimately to attain less than maximal expansion. Moreover, persistence of all the white stripes differs notably from the situation in many other white-spotted genotypes at this locus (e.g., $W/+$, $W^v/+$) and other loci (e.g., microphthalmia-white heterozygotes $Mi^{wh}/+$, or piebald s/s, or belted bt/bt), in which many of the "ghost" areas clearly become progressively invaded and obliterated by viable pigment cells. While the color intensity of the W^f/W^f viable clones is less than in the C3H wild type, this appears not to be due to a melanization deficiency, as the cells can be seen in culture to be fully pigmented (Fig. 2*a*).

Spontaneous Transformation and Tumorigenicity of Cultured W^f/W^f Melanocytes. Twenty-five of the explants from individual W^f/W^f mice failed to survive; this number greatly exceeds our failures with any other genotype (data not shown), thereby suggesting some growth disadvantage. Of the 10 remaining W^f/W^f explants, from which culture lines were established, 3 underwent transformation and were designated lines 1, 2, and 3 (Table 1). Lines 1 and 2 at first appeared to be normal (Fig. 2*a*) and then showed similar changes starting soon after the first split, at 15 wk and 8 wk, respectively. Coherent clusters of rounded cells began to detach from the dish and to grow in suspension (Fig. 2 *b* and *c*). Such clusters have been reported in some other transformed cell lines, including a human melanoma line (23). Some cells of W^f/W^f lines 1 and 2 failed to form clusters and, when isolated and clonally propagated, remained untransformed. Eight to 11 wk after clusters appeared, they started to reattach to the dish, spreading out to form a monolayer (Fig. 2*d*), which was easily removed by light trypsinization. Harvesting and replating of clusters invariably resulted in reattachment of the cells. The initial phase was termed stage **a**; the phase of suspended cell clusters, stage **b**; and the reattached phase, stage **c**. Transmission electron microscopy revealed a few advanced-type melanosomes in stage **b** cells, where they tended to be clumped, and dramatic differences from stage **a** to stage **c** (Fig. 2 *e* and *f*). Starting in stage **b**, mitochondria and free ribosomes were more numerous, endoplasmic reticulum and melanosomes were less abundant, and the cells by late stage **c** were relatively undifferentiated. Measurements documented a decline in melanin content from 230 pg per cell in stage **a** to 8 pg per cell in early stage **c** (Fig. 3). This was accompanied by decreased expression of tyrosinase-specific mRNA (data not shown). In further tests, the cells were found to be PMA-dependent in stage **a** but not in stages **b** and **c**; they also then grew in medium lacking PMA and containing only 2% instead of 20% fetal calf serum. Cells in stage **c** (but not in stage **a**) formed colonies in soft agar. When grown to confluence on plastic, stage **c** (but not stage **a**) cells formed foci (Fig. 4). Chromosome counts of stage **c**

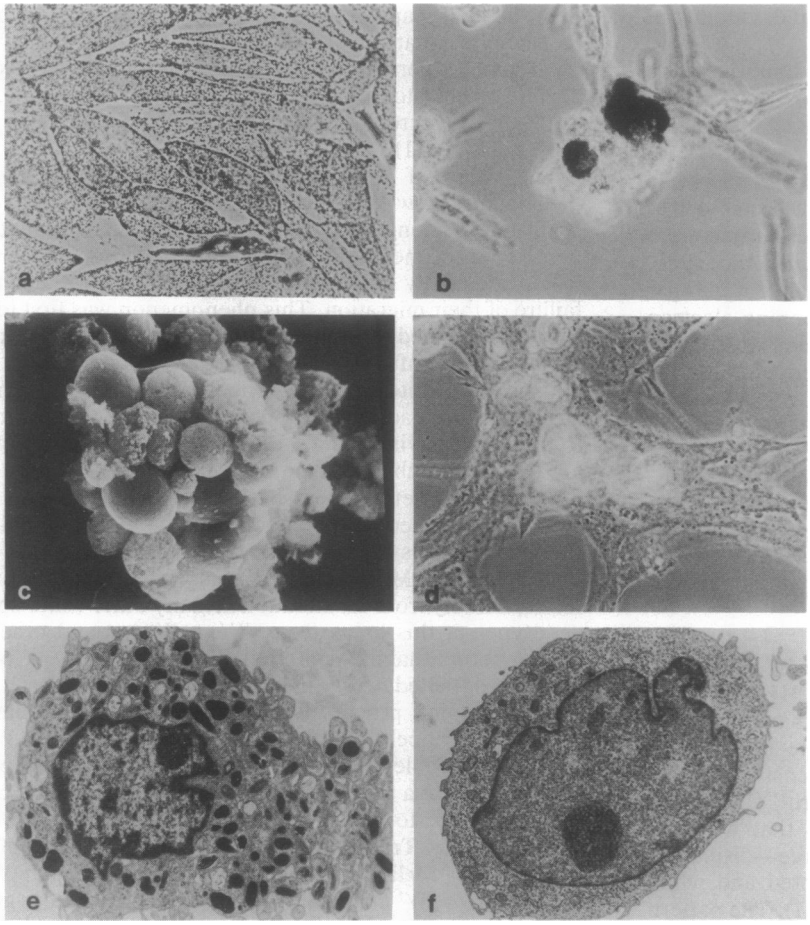

FIG. 2. Progressive transitions in lines 1 and 2 of cultured W^f/W^f melanocytes. (*a*) An original culture (stage **a**), with many melanin granules visible by phase contrast. (*b*) Detached clusters of cells (stage **b**) with generally decreased melanization. A few unusual dark cells are present. (*c*) Scanning electron micrograph of a cell cluster. (*d*) A cluster reattaching to the dish and growing out to form a layer of cells with little pigment (stages **b–c**). (*e* and *f*) Transmission electron micrographs showing changes from stage **a** to stage **c**, respectively. (*a*, *b*, and *d*, ×90; *c*, ×720; *e* and *f*, ×5400.)

cells of both lines 1 and 2 showed a modal number of 41 chromosomes. After subcutaneous injection in nude mice, stage **c** cells of each of these lines formed poorly differentiated malignant tumors of prevailing spindle-cell type that invaded the subjacent host muscle and were compatible with melanoma (Fig. 5). One tumor and cultured cells from the same line were stained with antiserum (Biomeda, Foster City, CA) for S-100 reactivity (often associated with melanomas) and were positive. Line 3 differed from lines 1 and 2 in that no cell clusters were formed. Six weeks after explantation, the cells became less dendritic and more epithelioid, began to lose melanin, and were more easily trypsinized. The ongoing attached-cell cultures grew in medium containing low serum and lacking PMA and formed foci at confluence. Doubling times of all three transformed cell lines were much shorter than that of the C3H parent line (Table 2).

In contrast, all cell lines from 1 $W^f/+$, 2 $W^v/+$, 2 $W/+$, 3 C3H, and 3 C57BL/6 explants and 41 independent examples of cell lines of 9 other genotypes remained untransformed. Fibroblast cell lines originating from skin of 2 W^f/W^f day-old mice were established in DMEM containing 10% fetal calf serum and also remained untransformed.

Molecular Analysis of the *W^f/Kit* Gene. As the W^f gene had not previously been isolated, we were interested in learning whether its structure might provide a clue to the tumorigenic

Table 1. Summary of tests for transformation of W^f/W^f cultured melanocyte lines

Criterion	Line 1	Line 2	Line 3
Morphologic change	+	+	+
Decreased doubling time*	+	+	+
Decreased melanin*	+	+	+
PMA-independence	+	+	+
Growth in low serum†	+	+	+
Cloning in soft agar	+	+	NT
Focus formation	+	+	+
Tumor formation‡	10/10	10/10	ND

NT, not tested; ND, no data available yet.
*Doubling time and melanin concentration were evaluated relative to coisogenic wild-type lines.
†This medium contained 2% fetal calf serum and also lacked PMA.
‡Tested by subcutaneous inoculation of ≈2 × 10^6 cells into nude mice. Twenty-four additional hosts were inoculated with melanocytes from cultured lines of $W^f/+$, $W^v/+$, and $W/+$ mutant genotypes and of C3H and C57BL/6 controls; all were negative. The other tests listed were also negative in these genotypes.

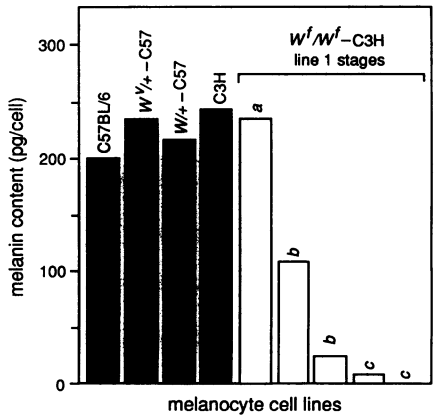

FIG. 3. The melanin content of a W^f/W^f transformed line of melanocytes (line 1) is at first (stage **a**) similar to that of the normal background strain (C3H) and the other genotypes shown but decreases progressively when cell clusters are formed (stage **b**) and when the cells reattach (stage **c**).

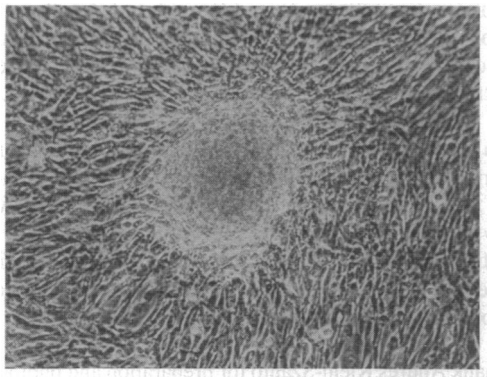

FIG. 4. Phase-contrast micrograph of focus formation at confluence in W^f/W^f transformed melanocytes (line 1, stage c). (×120.)

potential of some homozygous melanocytes. No obvious genomic rearrangements of the *Kit* gene were detected in Southern blot analysis of tissues from W^f/W^f mice (data not shown). The *Kit* cDNA was therefore isolated and sequenced to identify any point mutations or other small alterations. The coding sequence was divided into three overlapping segments for which three sense and anti-sense primers were prepared. RNA (10–20 μg) from melanocyte cell lines and from placenta was reverse-transcribed by using one of three antisense primers and Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) under conditions recommended by the manufacturer. A portion of the reaction (one-sixth to one-third) was amplified by 30 cycles of PCR (24) by using the reverse-transcription reaction primer and the appropriate upstream sense primer. A second amplification reaction, with an internal primer, was sometimes required to generate sufficient product. In these cases, the products of the first reaction were purified by using Geneclean (Bio 101, La Jolla, CA). The final PCR products were digested with *Hin*dIII and *Eco*RI, the recognition sites of which were incorporated at the 5′ ends of the oligonucleotides. These fragments were then cloned in pBluescript II (SK+) and sequenced. The oligonucleotides used (and in parentheses the positions to which they correspond) are as follows (with added restriction sites underlined): sense primers (3–24) 5′-GGCAAGCTTGCTCAGAGTCTAGCG-CAGCCAC-3′, (1088–1109) 5′-GGCAAGCTTATGAACAG-GACCTCGGCTAACA-3′, and (1981–2002) 5′-GGC-AAGCTTTATTGTGAACCTGCTTGGCGCA-3′; antisense primers (1163–1142) 5′-GGCGAATTCGGTTCACATATC-TGATGTTGCT-3′, (2043–2022) 5′-GGCGAATTC-CAATATTCTGTAATGACCAGGG-3′, and (2988–2967) 5′-GGCGAATTCAGCAGCAAAGCCTGTTGGACTT-3′.

Only one nucleotide difference was found between the wild-type C3H cDNA and that of W^f/W^f: An A → T substitution at position 2474 (numbered as in ref. 25), which converts codon 816 from AGG (Arg) to TGG (Trp). This alteration was confirmed in three independent reverse-transcription and amplification reactions from placenta and melanocyte sources. Three additional differences were detected in Kit cDNAs from both C3H and W^f/W^f-C3H mice as compared with the published sequence from BALB/c mice (25). Two changes (A → C at position 648 and C → T at 1618) were also noted by others (26), who sequenced various *W* alleles on the C57BL/6 background. A third change, a CG → GC inversion at positions 2358–2359, converts Ala-777 to glycine. This residue is also glycine in v-*kit* (27).

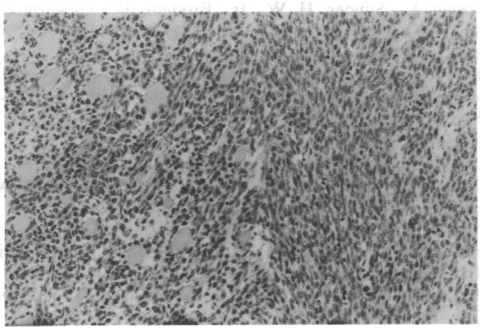

FIG. 5. Malignant tumor invading muscle of a nude host injected with cultured W^f/W^f melanocytes of line 1, stage c. (×65.)

Table 2. Doubling times of individual transformed W^f/W^f and untransformed melanocyte lines when the growth plateau was reached

Genotype*	Doubling time, hr	Time after explant, wk
W^f/W^f-C3H line 1†	35	56
W^f/W^f-C3H line 2†	30	44
W^f/W^f-C3H line 3†	38	36
W^f/W^f-C3H	135	34
$W^f/+$-C3H	200	24
+/+-C3H	168	26
$W^v/+$-C57BL/6	65	37
$W/+$-C57BL/6	72	36
+/+-C57BL/6	68	52

*Additional examples of the same genotypes were 6 W^f/W^f-C3H, 2 +/+-C3H, 1 $W^v/+$-C57BL/6, 1 $W/+$-C57BL/6, and 2 +/+-C57BL/6 melanocyte lines. Their doubling times ranged from 65 to 105 hr after 24–40 wk in culture and were therefore within the range of the other untransformed lines listed.
†Transformed lines.

Analysis of Kit mRNA in cultured W^f/W^f melanocytes by slot blot (Fig. 6) and northern blot (data not shown) revealed expression in untransformed (stage a) cells but virtual disappearance after transformation (stage c); β-actin mRNA was used as an internal control and did not diminish in stage c (data not shown). Expression of *Kit* occurred in untransformed melanocyte lines of other genotypes, including wild-type C3H and $W/+$-C57BL/6, and in wild-type C57BL/6 skin.

DISCUSSION

The Kit protein consists of an extracellular ligand-binding domain, a transmembrane segment, and a C-terminal intracellular tripartite domain with tyrosine-specific protein kinase activity (6, 25). Binding of the ligand initiates signal transduction to the cell nucleus. Oncogenic changes in such protooncogenes may involve various truncations and point mutations that constitutively activate the kinase and deregulate signaling (28). The W^f mutation reported here at position 816 of the Kit protein occurs in the central core of the

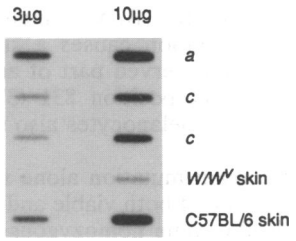

FIG. 6. Slot-blot analysis of Kit mRNA expression. Total RNA was isolated, and the blot was hybridized with a Kit Riboprobe. Kit is expressed in W^f/W^f melanocytes at stage a, but expression after transformation (stage c) decreases to a very low level. C57BL/6 skin is strongly positive; slight reactivity of W/W^v skin is attributable to some cells other than melanocytes.

conserved catalytic domain of the kinase (29), but position 816 is not itself highly conserved. It is located six residues C-terminal to the invariant residues implicated in ATP binding and five residues N-terminal to a putative autophosphorylation site at residue 821 (25). The kinase activity of the changed molecule was not measured, and the primary consequence of the change is unknown. It is thus not yet possible to determine the physiological basis for the spontaneous malignant transformation of W^f/W^f melanocytes in 30% of the lines established in culture. Nevertheless, the occurrence of malignant conversion is an unexpected consequence of a mutation initially impairing, rather than stimulating, cell growth; and it may provide a model to facilitate detection of other examples.

Melanocytes ordinarily divide very little *in vivo* postnatally but undergo increased proliferation under certain conditions and in culture. The failure of an unusually large number of W^f/W^f explants to survive in comparison with many other genotypes of melanocytes indicates a limited capacity to grow under the same conditions. A growth limitation *in vivo* is also inferred from the sustained failure of the pigmented (viable) melanocyte clones to occupy the spaces vacated prenatally by neighboring inviable ones, despite intermingling at the clonal borders (Fig. 1). Thus, it is not surprising that no obvious melanomas have been noticed in W^f/W^f mice. As *Kit* is normally expressed before and after birth in pigment cells (30), a mutation affecting expression could in fact influence many aspects of pigment cell growth, development, and survival. It is also of interest that germ cells, in which *Kit* is expressed at certain stages (30), are partly deficient in W^f/W^f mice despite full fertility of the animals. When W^f/W^f males were examined histologically, the number of gonocytes was subnormal at birth (31)—a mild version of the complete failure of early primordial germ cells to proliferate and survive in mice of more deleterious and sterile *W* mutant genotypes (3, 32).

The very early appearance of changes in the transformed cultures—after the first split—could result from prompt selection for those cells able to activate more efficient pathways for growth and mitogenesis. The phorbol ester PMA or other undefined factors in the placental extract or serum in the starting medium may play a role. PMA acts as a growth factor for cultured melanocytes and does not ordinarily transform them but is not required after transformation (13, 14). Nevertheless, it can act as a weak promoter, enhancing the carcinogenic effect of a weak initiator by binding to and activating a membrane-associated receptor. By activating protein kinase C, it can induce transcription of a number of protooncogenes and affect cell growth (33, 34). Yet a decrease in PMA binding and down-regulation rather than activation of protein kinase C has been noted in a PMA-dependent line of nontumorigenic murine melanocytes (35).

The W^{41}/W^{41} mutant mouse resembles the W^f/W^f mouse in many ways. It has a mild anemia and is fertile; though described as "mottled" (36), its coat pattern is in fact striped similarly to that of W^f/W^f (B. M., unpublished observations). As the W^{41} mutation causes a single amino acid change—also in a nonconserved part of an otherwise conserved kinase region—at position 831 (37), it will be of interest to learn if these melanocytes also sometimes transform in culture.

The W^f kinase-domain mutation alone is unlikely to account for the production of both viable and inviable melanoblast clones within the same homozygous individuals. The chinchilla-mottled (c^m/c^m) homozygous mouse with a mutation at the tyrosinase locus may serve as a paradigm. In this case, variable gene expression results in dark-grey and light-grey transverse stripes. We have found that the coding region of the gene is normal. However, a rearrangement is present upstream of the coding region that affects the chromatin structure and results in two different mitotically stabilized levels of transcription of the locus, each subnormal and each in half of the melanoblast clones (38). Whether there is also an upstream defect in the W^f gene is not yet known. Unlike c^m/c^m, where melanocytes of both phenotypes can be isolated in culture, the inviable W^f/W^f clones disappear prenatally, and their *Kit* expression cannot be tested. On the c^m/c^m model, W^f/W^f may have two different levels of *Kit* gene activity in melanoblast clonal initiator cells and their respective mitotic progeny. The low-expressing clones, deficient in both the level and specific activity of the kinase, would expire on an orderly programmed schedule.

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