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Phenotypic Reversions at the *W/Kit* Locus Mediated by Mitotic Recombination in Mice†

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The mouse *W* locus encodes Kit, the receptor tyrosine kinase for stem cell factor (SCF). Kit is required for several developmental processes, including the proliferation and survival of melanoblasts. Because of the nearly complete failure of W^{rio}/+ melanoblasts to colonize the skin, the coats of W^{rio}/+ mice are characterized by a majority of white hairs interspersed among pigmented hairs, giving a roan effect. However, 3.6% of W^{rio}/+ mice exhibit phenotypic reversions, i.e., spots of wild-type color on their coats with an otherwise mutant phenotype. Melanocyte cell lines were derived from each of six independent reversion spots on the skin of (C57BL/6 × DBA/2)F_1 W^{rio}/+ mice. All six melanocyte cell lines exhibited the general characteristics common to normal, nonimmortal mouse melanocytes. Of these, three revertant cell lines had lost the dominant-negative W^{rio} allele following mitotic recombination between the centromere and the *W* locus. One of the cell lines remained W^{rio}/+ but showed (i) stimulation in response to SCF and (ii) increased Kit expression, suggesting that the W^{rio} mutation can be rescued by increased endogenous expression of the c-*kit* proto-oncogene. Finally, two cell lines showed no detectable genetic change at the *W/Kit* locus and failed to respond to SCF stimulation in vitro. These results demonstrate that mitotic recombination can create large patches of wild-type hair on the coats of W^{rio}/+ mutant mice. This shows that mitotic recombination occurs spontaneously in normal healthy tissue in vivo. Moreover, these experiments confirm that other mechanisms, not associated with loss of heterozygosity, may account for the coat color reversion phenotype.

Reverse mutation is a rare event in mammals. Consistent with this rule, no reversion of the single base pair substitutions responsible for the mutations albino (*c*) and brown (*b*) was observed in studies involving more than 3 million homozygote mice (23, 47, 51). However, 12 mutations which affect pigmentation in mice show unusual phenotypic instabilities. Ten of these mutations are dominant, namely, viable-yellow (A^{vy}), varitint-waddler (*Va*), microphthalmia white (Mi^{wh}), and seven alleles at the *W/Kit* locus (W^{a}, W^{J2}, W^{37}, W^{42}, W^{ei}, $W^{v} = W^{55}$, W^{rio}). Two are recessive: pink-eyed unstable (p^{un}) and pearl (*pe*). Heterozygotes for a dominant unstable mutation, as well as homozygotes for an unstable recessive mutation, occasionally harbor wild-type pigmented spots on their otherwise mutant coats. These wild-type areas are not symmetrical and appear largely at random. In some cases, reversion events may be recovered in the germ line (43, 46). Various not mutually exclusive explanations could be considered to account for these phenotypic instabilities, including the possibilities that (i) the unstable mutation is not a single nucleotide pair substitution but rather a gene change associated with a high reversion rate, (ii) the mutant phenotype allows selection for a rare genetic event that would not be detected on the wild-type coat, and (iii) a nongenetic factor influences the expression of the mutant genotype and is responsible for what appears to be a functional mosaicism. The instability of the recessive pink-eyed unstable (p^{un}) mutation provides an example of the first mechanism. Indeed, it has recently been shown that reversion of p^{un} results from the occasional loss of a 70-kb duplication responsible for the mutation (14). The phenotypic instability of several alleles at the *W/Kit* locus may represent an example of the second and third mechanisms (8, 37; this report), while the de novo methylation of genes on the inactive X chromosome illustrates the third proposed mechanism.

We are interested in understanding the molecular bases of the high frequency of phenotypic reversion at the *W/Kit* locus in mice (for a review of Kit and its ligand, see reference 3). A previous genetic study has revealed that while some reversions observed on the coats of W^{ei}/+ heterozygous mice appeared to be coupled with loss of heterozygosity (LOH) at *buff*, a locus which lies 21 centimorgans distal from the *W* locus on chromosome 5, others were independent of such LOH (39). This was also consistent with our finding that pure cultures of melanocytes derived from independent reversion spots on the coats of C57BL/6 W^{ei}/+ mice were still heterozygous at the *W* locus and expressed both the mutated and the wild-type c-*kit* mRNAs (8). To investigate further the role of LOH in the appearance of wild-type spots on the coats of *W* mutant mice, we have studied melanocyte cell lines derived from reversion spots on (C57BL/6 × DBA/2)F_1 mice heterozygous for the W^{rio} allele. The W^{rio} allele was chosen since its deleterious effect on hair pigmentation is not lowered into the hybrid C57BL/6 × DBA/2 background, in contrast to W^{ei}. Such an influence of genetic background on the extent of white spotting in *W* mutant mice has been discussed extensively (47). Thus, (C57BL/6 × DBA/2)F_1 W^{rio}/+ mice display mostly white coats on which the reversion spots are clearly apparent. We took advantage of the genetic polymorphism for microsatellite sequences between the C57BL/6 and DBA/2 inbred mouse strains to search for LOH or a large deletion in melanocytes from reversion spots. In this study, we found that several reversions at the *W* locus were associated with mitotic recombination between homologous chromosomes. Our data also provide further support for the idea that other mechanisms, not

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† Dedicated to the memory of Hubert Condamine.

associated with LOH, also account for the coat color reversion phenotype. These mechanisms could be a *W* suppressor mutation and/or an epigenetic correction of the melanoblast defect associated with the *W* mutation.

MATERIALS AND METHODS

Mice. W^{rio} is a mutant allele at the *W/Kit* locus which occurred spontaneously in the DBA/2 inbred strain maintained at the Fundaçao Oswaldo Cruz, Rio de Janeiro, Brazil. DBA/2 (W^{rio}/+) and C57BL/6 (+/+) mice were raised in our animal center. BALB/cByJ (*nu*/*nu*) mice were purchased from IFFA-CREDO.

Establishment of melanocyte cell lines. Melanocyte cell lines were derived from 6- to 9-day-old (C57BL/6 × DBA/2)F_1 W^{rio}/+ mice harboring reversion spots. The skin (dermis and epidermis) beneath the reversion spot was removed under sterile conditions, and skin melanocytes were explanted and plated onto mitomycin C-treated XB2 feeder cells (42) in supplemented Eagle's minimal essential medium with 10% fetal calf serum and 10 nM cholera toxin (ICN Biochemicals) as previously described (1). Phorbol 12-myristate 13-acetate (200 nM) was added after the first day of culture. Cells were further subcultured onto mitomycin C-treated XB2 feeder cells until passage 7, when feeder cells were omitted as they were no longer needed. At passages 10 to 12, depending on the cell line, single-cell clones were derived by limiting dilution under conditions previously described (1). Assays for transformation and tumorigenicity of the cell lines were done as previously described (27).

Proliferation assays. Melanocytes (5×10^4) were plated into 96-well cluster plates and cultured in supplemented Eagle's minimal essential medium with 10% fetal calf serum and phorbol 12-myristate 13-acetate in the presence or absence of 50 U of recombinant murine stem cell factor (SCF) (R&D Systems) per ml. After 3 days, the cultures were pulsed for 16 h with [^{3}H]thymidine at 10 μCi/ml (specific activity, 60 mCi/mmol). Cells were collected on fiberglass filters with an automated collector, and incorporated [^{3}H]thymidine was counted by liquid scintillation spectrometry. This experiment was repeated several times with five replicates per treatment.

Cell staining and flow cytometry. Indirect immunofluorescence experiments were performed with cells either unfixed or fixed for 5 min in 1% paraformaldehyde by using anti-Kit monoclonal antibody ACK2, which specifically binds the extracellular domain of the murine Kit receptor (34) and a goat anti-rat immunoglobulin G-fluorescein isothiocyanate conjugate (Caltag Laboratories). For flow cytometry analysis, cells were collected in phosphate-buffered saline lacking calcium and magnesium chlorides but containing 200 μg of EDTA per ml prior to labeling. The mean labeling intensity per cell was determined with a FACScan (Becton Dickinson).

Southern and Northern (RNA) blot analysis and signal quantification. Quantification analysis of c-*kit* hybridization signals was performed with each reversion cell line. A 1.2-kb *Pst*I mouse renin probe from pRn5-3 (38) was used as an internal control of the DNA amount, while a 0.6-kb *Xho*I-*Mlu*I c-*kit* probe (8) was used to quantify the number of c-*kit* genes. Filters originally hybridized with the c-*kit* probe were stripped and rehybridized with the renin probe. Hybridizations were done overnight at 65°C in 2% sodium dodecyl sulfate–0.45 M Na_2HPO_4 (pH 7.2)–1 mM EDTA–0.5% skim milk. Final washes of the membranes were done in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 65°C. The blots were first exposed to Kodak Biomax films. Then, the PhosphorImager system (Molecular Dynamics) was used to quantify the hybridizing intensity of bands from the blots by using the volume integration function. The hybridizing intensities of both the DBA/2 parent and revertant cell c-*kit* signals were normalized against the signals of the renin genes. The c-*kit* copy number of the revertant cell lines was assessed by comparing the normalized ratios of the reversion cell lines to that of the DBA/2 mouse. Northern blot analysis was performed as previously described (8).

Simple sequence length polymorphism (SSLP) genotyping. Genetic typing of SSLPs was performed by PCR followed by electrophoresis on 3% Metaphor agarose gels (FMC Bioproducts) and ethidium bromide staining. The SSLPs used in this study were *D5Mit1*, *D5Mit11*, *D5Mit24*, *D5Mit41*, *D5Mit55*, *D5Mit72*, *D5Mit75*, *D5Mit95*, *D5Mit99*, *D5Mit146*, *D5Mit184*, and *D5Nds2* (10). PCR was performed with 80 ng of genomic DNA in 50 mM KCl–10 mM Tris-HCl (pH 8.0)–1.5 mM $MgCl_2$–0.1% Triton X-100–200 μM deoxynucleoside triphosphates–100 nM each primer–1 U of *Taq* polymerase in a final volume of 25 μl. The thermocycling protocol was as follows: an initial denaturation step at 93°C for 5 min, followed by 35 cycles of 93°C for 40 s, 55°C for 40 s, and 72°C for 30 s.

As a polymorphic marker at the *W/Kit* locus, we amplified a $(CA)_n$ motif found in the c-*kit* promoter region of the mouse genome at positions −3476 to −3432 from the transcription start site (9). An upstream primer (CCATGAAGGCT GGAGATGGA) and a downstream primer (GCTCAAGGATGCTTTCAT TGC) were designated in the flanking sequences to amplify a 176-bp fragment of C57BL/6 genomic DNA. The amplification protocol was as indicated above, except for the following points: the annealing temperature was 60°C, the number of cycles was reduced to 30, the deoxynucleoside triphosphate concentration was reduced tenfold, and 0.25 μl of [α-^{32}P]dCTP (3,000 Ci/mmol) was added to each sample. Aliquots of PCR products were loaded onto a 12% nondenaturing polyacrylamide gel and visualized after electrophoresis by overnight exposure to Kodak Biomax film.

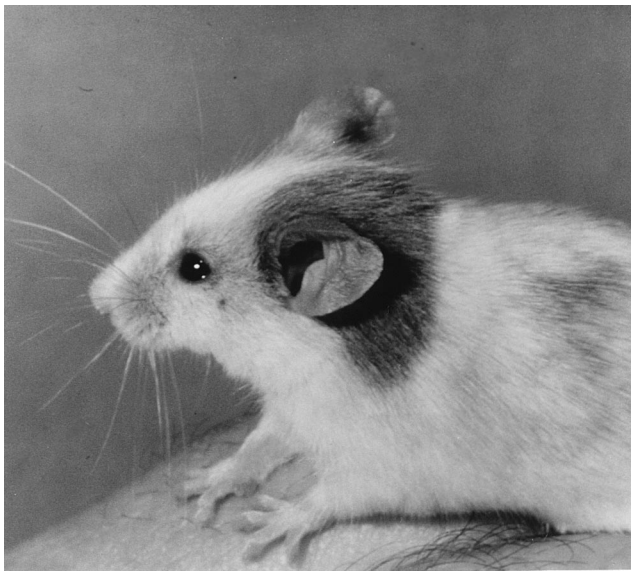

FIG. 1. Pigmentation pattern of a DBA/2 W^{rio}/+ mouse harboring a reversion spot.

RESULTS

Isolation and characteristics of melanocyte lines derived from reversion spots. Mice carrying the W^{rio} mutation have a pigmentation pattern resembling those associated with the severe W^{37}, W^{42}, and W^{ei} alleles: W^{rio}/+ heterozygotes are mostly white with few pigmented hairs, and homozygous individuals die late in gestation or immediately after birth of macrocytic anemia. In addition to the extensive white spotting, the W^{rio} allele is characterized by phenotypic instability in both the DBA/2 and (C57BL/6 × DBA/2)F_1 genetic backgrounds: 40 (3.6%) of 1,084 mice, hybrids between the C57BL/6 and DBA/2 inbred strains and heterozygous for the W^{rio} mutation, that is, (C57BL/6 × DBA/2)F_1 W^{rio}/+ mice, exhibited spots of intense pigmentation on an otherwise mutant coat nearly devoid of pigmented cells (Fig. 1). These spots are believed to result from the clonal growth of a melanocyte precursor having normal proliferative ability in a skin nearly devoid of melanoblasts (37). To dissect the molecular events responsible for the phenotypic instability of the W^{rio} mutation, skin melanocytes were explanted from reversion spots of six 6- to 9-day-old (C57BL/6 × DBA/2)F_1 W^{rio}/+ mice and cultured on mouse keratinocyte feeder cells. One melanocyte cell line was isolated from each of these independent cultures; these were designated mel-18, mel-23, mel-25, mel-27, mel-29, and mel-32.

Cultured melanocytes are considered normal when the cells are all visibly pigmented, often dendritic, contact inhibited, and unable to grow without phorbol 12-myristate 13-acetate or the equivalent, and do not form tumors when used to inoculate animals (1, 2, 27, 28, 49). These properties were tested on the cell lines from reversion spots. All six populations contained only pigmented cells with a dendritic shape and were similar in appearance (Fig. 2A). Furthermore, all of these melanocyte lines were growth inhibited at confluence and remained dependent on both phorbol 12-myristate 13-acetate (200 nM) and a high concentration (10%) of fetal calf serum for growth. In addition, none of the lines proliferated in soft agar or formed any transient nodule or tumor when grafted into athymic (*nu*/*nu*) mice. Furthermore, because it has been shown that tumoral progression is associated with loss of c-*kit* expression in human cutaneous melanoma (12, 29, 33), we tested c-*kit*

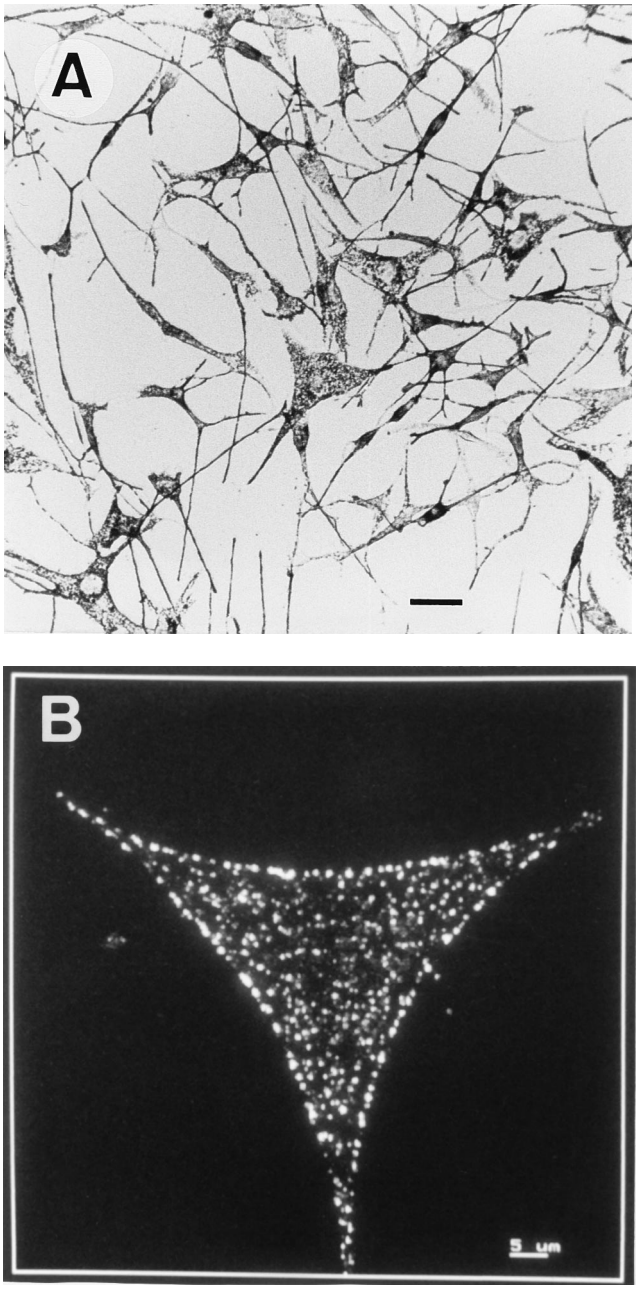

FIG. 2. Morphology of melanocytes derived from reversion spots. (A) Bright-field photomicrographs of a mel-25 culture. (B) Immunolocalization of Kit on the surface of a mel-18 cell. Melanocytes were plated on glass slides. Cells were fixed in 1% paraformaldehyde–phosphate-buffered saline and reacted with a monoclonal antibody that binds to the extracellular domain of the murine Kit receptor, followed by fluorescein isothiocyanate-conjugated second antibodies, and analyzed by laser scanning confocal microscopy. A and B are representative of the morphology and staining pattern of the six reversion cell lines. Bar in A, 10 μm.

expression in all six melanocyte cell lines. c-*kit* expression was found in all six lines, as demonstrated by Northern blot analysis (data not shown) and confirmed by immunofluorescence staining with ACK2, a monoclonal antibody specific for the extracellular domain of the murine Kit receptor (Fig. 2B). Hence, all six melanocyte cell lines from reversion spot explants failed to express any of the important phenotypic characteristics of melanocytes derived from premalignant and malignant melanocytic lesions (17, 20, 27). Therefore, these cells were considered to be immortal, nontumorigenic melanocytes.

Reversions did not result from c-*kit* gene dosage effects. An increased copy number of the wild-type c-*kit* gene or loss of the mutant allele might give a growth advantage to melanoblasts over genuine $W^{rio}/+$ melanoblasts. To assess the number of c-*kit* genes carried by the melanocyte cell lines derived from the reversion spots, we compared the signal response ratios produced by a c-*kit* probe and a probe for the renin genes, located on mouse chromosome 1, in the melanocyte cell lines, as well as in control DNA samples. The renin probe was chosen as a normalized signal since DBA/2 mice harbor two renin genes, *Ren1* and *Ren2*, while C57BL/6 mice carry only the *Ren1* gene (40). Thus, (C57BL/6 × DBA/2)F_1 $W^{rio}/+$ mice, from which all six melanocyte cell lines were derived, carry two copies of the *Ren1* gene but a single copy of the *Ren2* gene per diploid genome. Hence, the number of c-*kit* genes carried in each DNA sample may be readily compared to a single-copy sequence or to a double-copy sequence; either sequence hybridized to the same renin probe (Fig. 3).

Quantification of hybridization intensity at the *W*/*Kit* and *Ren1*/*Ren2* loci between the melanocyte cell lines derived from reversion spots and a normal DBA/2 mouse was conducted by using storage phosphor technology. Our data (Table 1) indicate that the hybridization intensity of the c-*kit* band was equivalent to that of the distinctive *Ren1* band in the melanocyte cell lines. By contrast, the intensity of the c-*kit* band relative to the distinctive *Ren2* band was twice as great. These results are consistent with the presence of two c-*kit* gene copies per diploid genome in all melanocyte cell lines, thus indicating that the melanocytes from reversion spots had no changes in c-*kit* copy number.

Loss of the W^{rio} allele in several revertant cell lines. To distinguish between homozygosity and heterozygosity at the *W*/*Kit* locus in the melanocyte cell lines, we took advantage of the presence of a dinucleotide $(CA)_n$ repeat in the c-*kit* 5′-flanking sequence (Fig. 4a). Primers made from unique sequences that bracket this repeat were used to define a polymorphism in the C57BL/6 and DBA/2 strains. Indeed, PCR-amplified c-*kit* fragments generated from the C57BL/6 and DBA/2 alleles have different sizes (Fig. 4b). This polymorphism allowed us to demonstrate that three melanocyte cell lines, namely, mel-18, mel-29, and mel-32, lacked the c-*kit* gene polymorphism characteristic of the DBA/2 allele, while the others (mel-23, mel-25, and mel-27) were still heterozygous at the *W*/*Kit* locus (Fig. 4b). Various genetic mechanisms could account for the LOH at the *W*/*Kit* locus observed in mel-18, mel-29, and mel-32 cells, including a chromosome nondisjunction with loss of the DBA/2 chromosome and reduplication of the remaining wild-type C57BL/6 chromosome, or an interchromosomal recombination.

Loss of alleles for loci on chromosome 5 in the mel-18, mel-29, and mel-32 revertant cell lines. To test these hypotheses, the chromosome 5 patterns of the revertant cell lines were typed by PCR with primer pairs flanking simple sequence repeats which serve as specific probes for either the C57BL/6 or the DBA/2 genome (10). Figure 5 indicates the location and the status, either heterozygosity or LOH, for 10 microsatellites for each melanocyte cell line. These data reveal that the allele losses at the *W* locus observed in mel-18, mel-29, and mel-32 cells were associated with LOH for several distal chromosome 5 markers, while several proximal markers remained heterozygous. Such a conformation implies that chromatids of the different homologs crossed over between the *W* locus and the centromere to produce both a c-*kit* (+/+) melanoblast responsible for the wild-type reversion spot and a pigment precursor

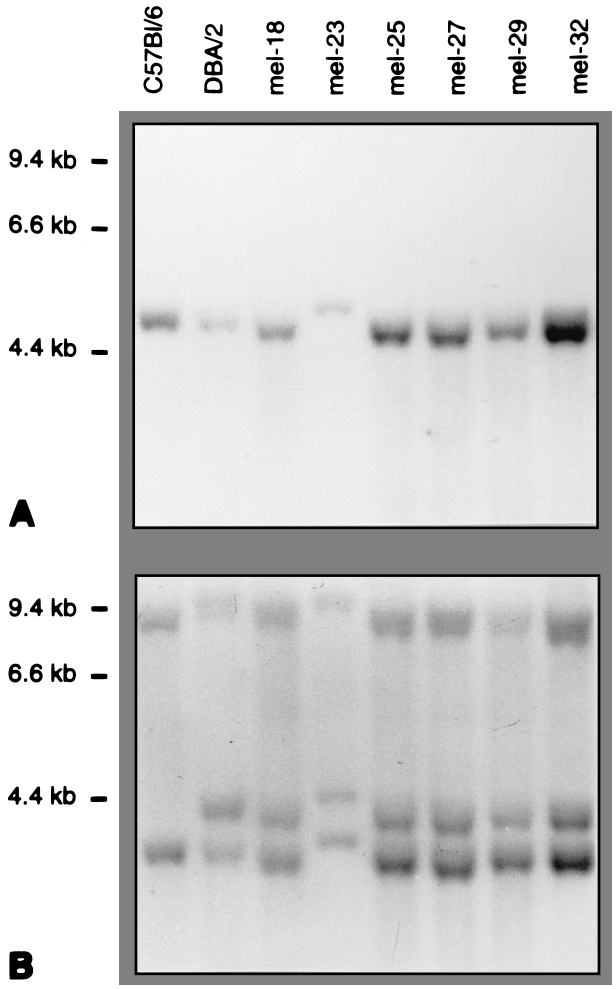

FIG. 3. Southern blot analysis of c-*kit* (A) and *Ren-1* and *Ren-2* (B) genes in C57BL/6 and DBA/2 mice and in the six melanocyte cell lines derived from reversion spots on the coats of (C57BL/6 × DBA/2)F_1 W^{rio}/+ mice. DNA samples from parent C57BL/6 and DBA/2 mice and from each melanocyte cell line were digested with *Eco*RI, electrophoresed in a 0.7% agarose gel, and transferred to Hybond-N+ membranes. Southern blots were hybridized successively with a c-*kit* probe (A) and a renin probe (B). DBA/2 mice harbor two renin genes per haploid genome, named *Ren1* and *Ren2*, while there is only one renin gene (*Ren1*) in C57BL/6 mice (41). *Eco*RI digestion produces four fragments of 3.9, 4.4, 8.8, and 9.2 kb hybridizing with the renin probe. The 3.9- and 4.4-kb renin *Eco*RI fragments, distinctive of the *Ren-1* and *Ren-2* genes, respectively, were used as internal controls of the DNA amount. The c-*kit* probe hybridized with a single 5-kb *Eco*RI fragment.

cell having a c-*kit* (W^{rio}/W^{rio}) genotype fatal to melanocytes. As deduced from the combinations of alleles, all crossings over occurred between the centromere and the *W* locus. Furthermore, the breakpoints are evenly distributed, as expected for products from independent mitotic recombination events. We conclude from these features that single mitotic recombination can account for mel-18, mel-29, and mel-32 reversion events. Furthermore, two C57BL/6 markers, namely, *D5Mit11* and *D5Mit55*, proximal to the point of mitotic recombination were lost in the mel-32 cell line; this suggests deletion of the corresponding C57BL/6 region. In addition, these data confirm that no LOH was associated with the reversion events responsible for the wild-type spots from which the mel-23, mel-25, and mel-27 cell lines were derived.

Effect of the Kit ligand on proliferation of revertant melanocytes in vitro. The stimulatory effect of the Kit ligand, SCF, on the growth of the melanocytes derived from the six reversion spots was examined. Melanocytes were cultured in the absence of feeder cells and either with or without SCF. After 3 days in culture, the mitogenic response to SCF was assayed by determining [^{3}H]thymidine incorporation as a measure of DNA synthesis. The results show that SCF significantly stimulated the proliferation of mel-18, mel-29, and mel-32 melanocytes in culture (Table 2), indicating that the Kit/SCF pathway was functional in mel-18, mel-29, and mel-32 cells, in agreement with the finding that these cells had become ho-

TABLE 1. Allelic copy numbers in melanocyte cell lines derived from reversion spots on (C57BL/6 × DBA2)F_1 W^{rio}/+ mice

Sample	Copy no. of c-*kit* gene relative to *Ren1* gene copy no.[a]	Copy no. of c-*kit* gene relative to *Ren2* gene copy no.[a]
DBA/2 mouse	1	1
mel-18	0.92	1.72
mel-23	0.95	1.75
mel-25	0.89	1.88
mel-27	0.81	1.57
mel-29	0.77	1.68
mel-32	1.02	2.14

[a] The intensities of c-*kit*, *Ren1*, and *Ren2* signals were calculated by using the corresponding DBA/2 signal intensity as a reference. The number of c-*kit* alleles relative to the *Ren1* and *Ren2* copy numbers was determined by dividing the c-*kit* intensity by the 3.9-kb *Ren1* fragment intensity and the 4.4-kb *Ren2* fragment intensity, respectively.

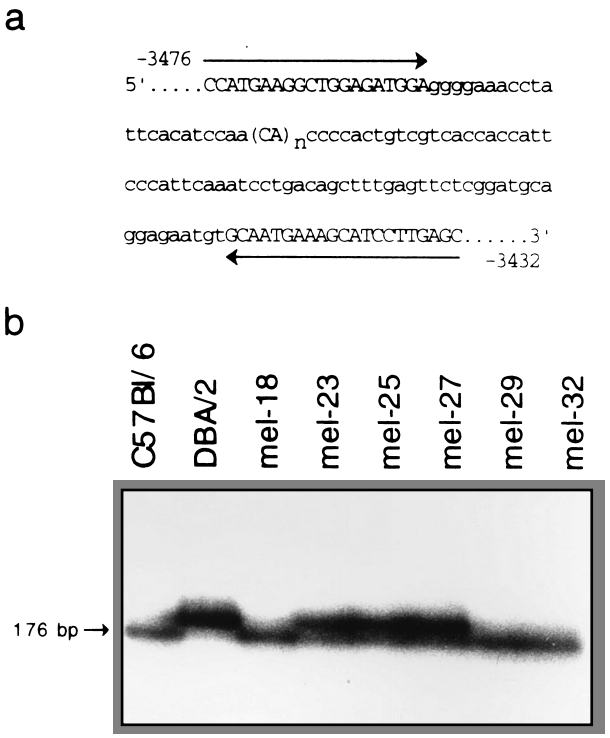

FIG. 4. Polymorphism at the *W/Kit* locus. (a) Amplified sequence in the c-*kit* promoter region. The numbers refer to the positions in the sequence relative to the transcription initiation site. Oligonucleotides that bracket the microsatellite $(CA)_n$ motif are indicated by arrows. In C57BL/6 DNA, the dinucleotide CA is repeated 22 times and the corresponding amplified fragment is 176 bp long. (b) SSLP detection after migration in a 12% polyacrylamide gel and autoradiography. The PCR-amplified DBA/2 fragment is slightly larger than the corresponding C57BL/6 product.

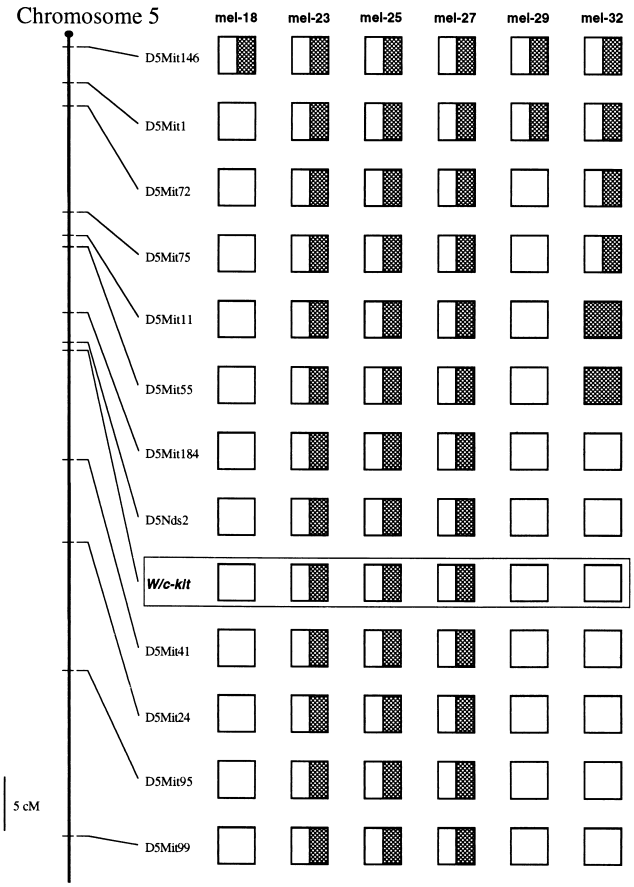

FIG. 5. Genetic analysis of melanocyte cell lines derived from six reversion spots on the coats of (C57BL/6 × DBA/2)F_1 W^{wio}/+ hybrid mice, identified as mel-18, mel-23, mel-25, mel-27, mel-29, and mel-32, with SSLP markers specific for mouse chromosome 5. The relative positions of SSLP markers are in accordance with the Whitehead Institute-Massachusetts Institute of Technology database. The *W/Kit* probe is boxed. cM, centimorgans. □, C57BL/6 allele; ▩, DBA/2 allele; ◧, heterozygous for the DBA/2 and C57BL/6 alleles.

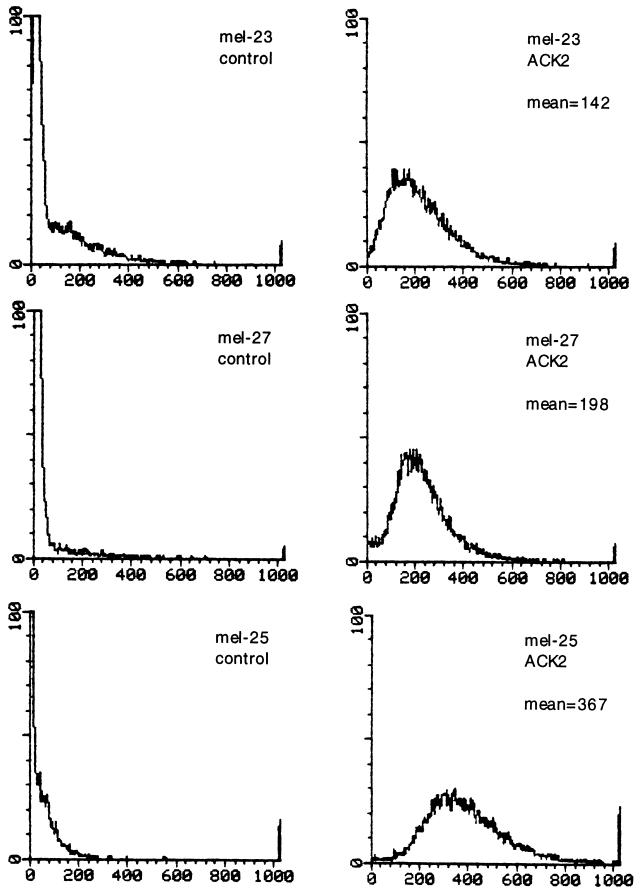

FIG. 6. Immunofluorescence analysis of Kit expression in melanocyte cell lines. Histograms show fluorescence intensity on the abscissa and cell number on the ordinate. Cells were successively incubated with Rat anti-Kit monoclonal antibody ACK2 and an anti-Rat immunoglobulin G-fluorescein isothiocyanate conjugate. As a control for the measurement of nonspecific binding of the fluorochrome, cells were also incubated only with the fluorescein isothiocyanate-labelled antibody. The mean value is the average fluorescence for the entire population and is corrected in accordance with the mean value of the control.

mozygous at the *W/Kit* locus. The observation that SCF triggers mouse melanocyte proliferation in vitro correlates with what has been reported on melanocyte precursors in quails (26), as well as on adult human melanocytes (12). By contrast, no significant effect of SCF on the proliferation of mel-23 and mel-27 melanocytes was found (Table 2), corroborating the fact that these cells remained heterozygous at the *W/Kit* locus.

TABLE 2. Effect of SCF on thymidine incorporation in melanocytes[a]

Cell line	Genotype	Radioactivity incorporated (cpm)		*P* value
		Control	SCF (50 U/ml)	
mel-18	+/+	5,972 ± 474	7,749 ± 666	<0.01
mel-23	+/*Wrio*	13,743 ± 522	13,445 ± 1,522	>0.1
mel-25	+/*Wrio*	24,872 ± 2,447	30,778 ± 1,773	<0.01
mel-27	+/*Wrio*	80,902 ± 6,670	85,583 ± 1,201	>0.1
mel-29	+/+	99,421 ± 7,511	120,190 ± 3,850	<0.01
mel-32	+/+	12,027 ± 733	14,596 ± 853	<0.01

[a] Cells were incubated for 3 days either with or without SCF and pulsed with [^{3}H]thymidine for the last 16 h. Each value is the mean of five samples ± the standard deviation. Statistical analysis was done with Student's *t* test.

Surprisingly, a significant effect of SCF on proliferation of mel-25 cells was observed (Table 2).

To test whether SCF responsiveness of mel-25 was related to the amount of the Kit receptor on the cell surface, flow cytometry analysis was conducted with growing cultures of mel-23, mel-25, and mel-27 cells by using anti-Kit monoclonal antibody ACK2. The mean amounts of the Kit receptor per cell, as measured by the labeling intensity, were comparable between mel-23 and mel-27 cells, whereas Kit expression was about twofold higher in mel-25 cells (Fig. 6). While these data only demonstrate a correlation between SCF response and increased Kit receptor expression in mel-25 cells, they are consistent with the possibility that overexpression of the endogenous c-*kit* proto-oncogene may restore the capacity of *W* mutant melanocytes to respond to SCF (see Discussion).

DISCUSSION

Mammalian chromosomes do not appear to pair during mitosis. However, LOH is frequently observed in premalignant and malignant tissues and has been used as a first step in positional cloning of tumor suppressor genes. Several mechanisms have been identified as responsible for this LOH, includ-

ing hemizygous deletion, mitotic nondisjunction with loss of an entire chromosome and reduplication, and mitotic recombination. The hallmarks of single mitotic recombination are the following: heterozygosity for at least one locus in the proximal region of the chromosome and homozygosity throughout the rest of the chromosome, including the targeted locus. By using these criteria, mitotic recombinations have been shown to have occurred spontaneously in retinoblastoma (7, 13, 52), rhabdomyosarcoma (45), ovarian cancer (50), and astrocytomas (24), as well as in skin carcinomas induced by chemical carcinogens (4, 5). Furthermore, mitotic interchromosomal recombinations have been shown to occur spontaneously in mammalian cells cultured in vitro (15, 19). Finally, the existence of mitotic recombination in normal, healthy tissue of mammals was also suspected (16, 31). We have previously predicted its existence to account for data obtained from genetic studies on *W* phenotypic instability (39). Our present analysis of melanocytes derived from reversion spots demonstrates that recombinations between two homologous chromosomes occur during normal embryogenesis in mammals. This implies that distinct cell lineages may be created during mammal development and lead to mosaicism in adult tissues. Furthermore, this result supports the premise that mitotic recombination can initiate tumors, such as retinoblastoma.

Nonetheless, the frequency of reversion spots in *W* mutant mice cannot be used as an indicator for the incidence of mitotic recombination in mutagenicity tests since several reversion events seen on *W* mutant coats are not associated with LOH at the *W*/*Kit* locus (8; this study). The fact that some melanoblasts, although heterozygous for a strong allele at the *W* locus and thus programmed to die, occasionally give rise to a high number of melanocytes responsible for reversion spots implies that *W* mutations can be rescued in vivo.

An open question is how such melanocytes acquired normal survival and proliferative capacities. In this study, two hypotheses were rejected. The *W*/*Kit* locus is sensitive to gene dosage, as discussed more fully below. Thus, duplication of the wild-type allele or chromosome 5 trisomy with two chromosomes of C57BL/6 origin could have resulted in the synthesis of a sufficient number of active Kit receptors to rescue the deficiency associated with the dominant-negative W^{rio} mutation. However, the three melanocyte cell lines heterozygous at the *W* locus still have two c-*kit* genes, including the mutated allele. Alternatively, it may be argued that such reversion spots result from colonization of the skin by the descendants of a transformed melanoblast. Nonetheless, the melanocyte cell lines derived from reversion spots exhibit none of the phenotypic characteristics associated with either preneoplastic melanocytes or melanoma cells.

Several other mutually nonexclusive explanations seem plausible. The first hypothesis comes from insight into the molecular bases for the dominant-negative mutations of the receptor (for a review see reference 41). It has been shown that strongly dominant c-*kit* mutations, such as W^{42} and W^{ei}, are the result of point substitutions that abolish tyrosine kinase activity (8, 35). Since the Kit receptor is presumed to dimerize in response to ligand binding, inducing activation of intrinsic tyrosine kinase activity, the mutant polypeptides are expected to be responsible for the formation of defective heterodimers in heterozygotes. Thus, if normal and mutant polypeptides reassort at random, up to 75% of potential dimers may be nonfunctional in $W^{42}/+$ and $W^{ei}/+$ melanoblasts; this reduction causes extensive white spotting. By contrast, the original *W* allele is a null allele (18, 35) which results in a 50% reduction in the cell surface receptor number in heterozygous melanoblasts; however, *W*/+ mice have only a limited pigmentation deficiency, i.e., a white belly spot, white feet, a white tail tip, and occasionally a small white head spot. These observations indicate that the severity of the coat color phenotype in the heterozygous state parallels the reduction in the amount of functional receptor molecules on the cell surface and that a 50% reduction in the amount of Kit is not sufficient to cause extensive white spotting. Accordingly, this model predicts that twofold overexpression of both the normal and mutant polypeptides in the same heterozygous cell should be able to restore the absolute number of active dimers per cell. In other words, a twofold increase of c-*kit* expression should suppress the mutant phenotype, provided that the amounts of ligand and intracellular substrates are not limiting. Our data do not give conclusive evidence that the reversion spot from which mel-25 was derived is secondary to the enhanced expression of both c-*kit* alleles in a melanocyte precursor, but they point in this direction.

Another possibility is that a mutation able to suppress the loss-of-function mutation occurred in a melanoblast precursor. Since the Kit function is still impaired in two melanocyte cell lines, as revealed by the fact that these failed to respond to SCF, this gain-of-function mutation would affect a gene whose product acts downstream from Kit in the signaling pathway and generate constitutive stimulation of the pathway. The existence of such gain-of-function mutations affecting genes acting in receptor tyrosine kinase signaling cascades and sufficient to activate the corresponding pathways has been confirmed in drosophila (6, 30).

Finally, another tyrosine kinase receptor that is normally not expressed in melanoblasts but is able to promote the survival and possibly the proliferation of melanoblasts in *W* mutant mice could be turned on occasionally. Several arguments support this view. First, *W* mutations exert deleterious effects on primordial germ cells, melanoblasts, interstitial cells of Cajal, and hematopoietic progenitors, and Kit is consistently expressed in each of these cells (21, 25). However, there is accumulating evidence for the presence of the Kit receptor in cells that are not affected in *W* mice (32, 36, 48). This suggests either that Kit/SCF has no function in these cells or, alternatively, that another signal transduction pathway substitutes for it in *W* mutant mice. Second, W^v/W^v mice are usually sterile. However, in the 129/Sv genetic background, these homozygotes are fertile (16a), indicating that the 129/Sv background may compensate for the gonadal defects due to the W^v mutation. Such a small degree of compensation may result from the expression of another receptor tyrosine kinase mimicking the Kit function in gonads. Third, and more significantly, the original *W* mutation is a single base substitution at the 5′ splice donor site of exon 10 of the c-*kit* gene (18); thus, no cell surface expression of Kit is detected on *W*/*W* mast cells (35). As a result, in most genetic backgrounds, *W*/*W* homozygotes suffer from severe macrocytic anemia and die perinatally. However, Russell and Lawson (44) have succeeded in obtaining *W*/*W* adults, implying that the *W* defect in hematopoiesis may be rescued in some genetic backgrounds, presumably by other signaling pathway. Fourth, c-*fms* and CSF-1, the receptor for the macrophage growth factor colony-stimulating factor 1 and its ligand, are able to complement in vitro the defect in mast cells derived from *W*/*W* mutant mice (11), while the *ret* gene product can partially compensate for the defect of melanoblasts when introduced in the germ line of W^v/W^v mice by means of transgenesis (22). These lines of evidence, although not conclusive, provide strong support for the hypothesis that another receptor tyrosine kinase signaling pathway is able to compensate for the loss of Kit-SCF action in several tissues affected by *W* mutations.

Nonetheless, it remains possible that, as discussed above, overexpression of c-*kit* and/or dominant mutations responsible for the deregulation of Kit signal transduction events also account for reversion spots on the coats of *W* mutant mice. Clearly, more remains to be learned about the Kit signaling pathway before we understand the mechanism of phenotypic reversion in cases in which no mitotic recombination is involved.

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