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Deletion of the c-*kit* protooncogene in the human developmental defect piebald trait

(pigmentation disorders/melanocytes/chromosome deletion/platelet-derived growth factor receptor α/melanoma growth-stimulatory activity)

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ABSTRACT **The protooncogene c-*kit* is critical for development of hematopoietic stem cells, germ cells, and melanoblasts in the mouse. Homozygous mutations of this gene in the mouse cause anemia, infertility, and albinism, whereas heterozygous mutant mice usually exhibit only a white forehead blaze and depigmentation of the ventral body, tail, and feet. The heterozygous mouse phenotype is very similar to human piebald trait, which is characterized by a congenital white hair forelock and ventral and extremity depigmentation. To investigate the possibility that alterations in the human c-*kit* gene may be a cause of piebald trait, DNA from seven unrelated affected individuals was examined by Southern blot analysis. One subject, although cytogenetically normal, has a heterozygous deletion of the c-*kit* protooncogene. This deletion encompasses the entire coding region for c-*kit* and also involves the closely linked gene for platelet-derived growth factor receptor α. Fluorescence *in situ* hybridization of genomic c-kit probes to metaphase chromosomes independently confirmed the deletion in this case. These findings provide molecular evidence mapping piebald trait to the c-*kit* locus on chromosome 4. Although we cannot exclude the involvement of other closely linked genes, the demonstration of a genomic c-*kit* deletion in one subject with piebald trait and the marked concordance of the human and mouse phenotypes provide strong evidence for the role of c-*kit* in the development of human melanocytes and in the pathogenesis of piebald trait.**

Congenital white spotting syndromes in both animals and humans are thought to result from a failure of embryonic migration or proliferation by primitive melanoblasts (1, 2). One of these disorders, mouse "dominant spotting," results from mutations in the protooncogene c-*kit* (3, 4). In heterozygous mice the manifestations of this mutation are generally limited to a white forehead blaze and areas of depigmentation on the ventral body, tail, and feet (5). Homozygous mice, however, exhibit a complete absence of skin and hair pigmentation, severe macrocytic anemia, and infertility, indicating that the function of this tyrosine kinase membrane receptor is necessary for normal development of melanocytes, hematopoietic stem cells, and germ cells in the mouse (6). Although the pattern of tissue-specific expression in humans is similar to the mouse (7–9), a parallel role for c-*kit* in human development has not been demonstrated.

One approach to understanding c-*kit* receptor function in human development is to examine human white spotting syndromes that phenotypically resemble mouse dominant spotting for abnormalities in this gene. Indeed, one disorder, human piebald trait, is very similar to mouse dominant spotting and is characterized by autosomal dominant inheritance, a white hair forelock, stable areas of hypopigmentation or "leukoderma" on the anterior chest and extremities, and the absence of extracutaneous manifestations (10). In addition, cytogenetic data have implicated the long arm of chromosome 4, a region that includes the human c-*kit* gene (7, 11, 12), in the pathogenesis of piebald trait in three children with large interstitial deletions and multiple congenital anomalies (13).

In this paper, we report initial studies of seven piebald individuals from unrelated kindreds for alterations in the human c-*kit* gene. Using both Southern blot analysis and fluorescence *in situ* hybridization, we show that one of these subjects, although cytogenetically normal, has a heterozygous deletion of the c-*kit* gene. The deletion encompasses the closely linked gene for the platelet-derived growth factor receptor α (PDGFRα) (14–16) but not the gene for melanoma growth-stimulatory activity (MGSA, also known as *gro*) (17, 18), which has been suggested as a candidate gene for piebald trait (18). The results provide molecular data mapping piebald trait to the human c-*kit* gene on chromosome 4q11–12. In addition, the data suggest that mutations in the human c-*kit* gene can cause piebald trait and that this membrane receptor has a similar function in both human and mouse development.

MATERIALS AND METHODS

Subjects. Seven piebald individuals, each from unrelated kindreds, were studied. In all cases the hair and skin findings were present from birth. Subject A is a nulliparous Caucasian woman with a white forelock and extensive white patches on the abdomen and lower legs. She has no family history of piebald trait. Subject B is a Hispanic man who was born with a small white forelock that became less perceptible with time and persistent areas of leukoderma on the lower legs and anterior chest. Although his parents appear to have been unaffected, his daughter also has piebald trait (Fig. 1*A*). Subject C is a Black woman with a white forelock and no leukoderma; she had a similarly affected mother and maternal grandmother. Subject D is a native American woman with a marked white forelock, depigmentation of the forehead that extends to the eyebrows, and extensive areas of depigmentation that spare only the back. Her only child has the same phenotype. Subject E is a Caucasian male with a white hair forelock, leukoderma, and two similarly affected sons. Subject F is a Caucasian male with a small area of leukoderma on the midabdomen and a long family history of white hair forelock and leukoderma consistent with autosomal dominant transmission in at least three generations. Subject G is a Black male infant born with a white forelock and extensive areas of leukoderma on the abdomen, arms, and legs but with no prior

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Abbreviations: PDGFRα, platelet-derived growth factor receptor α; MGSA, melanoma growth-stimulatory activity; IL-2, interleukin 2.
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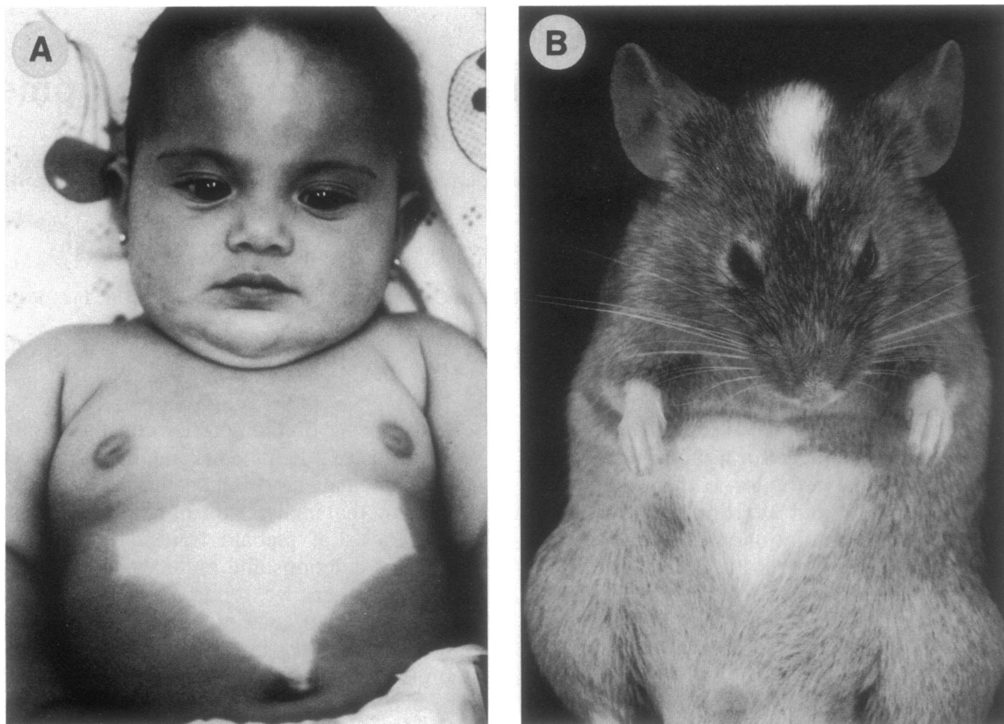

FIG. 1. Cutaneous manifestations in a patient with piebald trait (*A*) compared with a mouse with dominant spotting (*B*) resulting from a mutation in one allele of the c-*kit* protooncogene (*W* locus). Note that both species manifest areas of hypopigmentation on the midforehead and on the ventral abdomen in the midline. The father of the child shown is subject B (see text), who has similar findings of piebaldism and was the only family member from whom DNA could be obtained. The mouse is a C3H/HeJ male of the $W^x/+$ genotype.

family history. None of the subjects had extracutaneous manifestations that would suggest the diagnosis of the complex disorder Waardenburg syndrome, which also has pigmentary disturbances and maps to human chromosome 2 (19).

Probes. The c-kit cDNA probes, obtained from the American Type Culture Collection, are the human cDNA (phckit-171) (7) and the cat v-kit probe (pv-kit) (12). The genomic c-kit probes G12p and G30p (20) were provided by C. deCastro (Duke University, Durham, NC). The probe to the cytoplasmic domain of PDGFRα (14) was provided by A. Sakaguchi (University of Texas at San Antonio) with permission from S. Aaronson (National Cancer Institute, Bethesda, MD). The probe for human interleukin 2 (IL-2) (psvIL2) (21) was provided by L. Davis (University of Texas Southwestern, Dallas) with permission from G. Crabtree (Stanford University, Palo Alto, CA). The probe for the human MGSA gene (pGEMTC870) (17) was a gift of R. Sager (Dana-Farber, Boston, MA). The cDNA inserts were isolated from the plasmids by digestion with the appropriate restriction enzymes, followed by electrophoresis and electroelution from the agar slice containing the cloned insert.

Southern Blot Analysis. Blood was obtained with informed consent and genomic DNAs were prepared from lymphoblastoid cell lines obtained by Epstein–Barr virus immortalization of peripheral blood lymphocytes. DNAs were digested with restriction endonucleases as recommended by the manufacturer (Boehringer Mannheim) and separated by electrophoresis in a 0.7% agarose gel. After electrophoresis, the gel was treated with 0.2 M HCl for 15 min, rinsed several times with water, and then soaked in several volumes of denaturing solution (1.5 M NaCl/0.5 M NaOH) for 1 hr at room temperature, followed by neutralization in 1 M Tris·HCl, pH 8.0/1.5 M NaCl. After transfer to nitrocellulose, the membrane was baked at 80°C for 2 hr. Prehybridization was performed overnight at 42°C in 0.02 M Tris·HCl, pH 7.4/5× standard saline citrate (SSC)/5× Denhardt's solution/50% (vol/vol) formamide/denatured salmon sperm DNA (50 μg/ml). cDNA probes were labeled with [α-^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq; Amersham) by random priming according to the manufacturer's instructions (Boehringer Mannheim) to a specific activity of 10^8 cpm/μg, and hybridization was carried out for 18–24 hr at 42°C in hybridization buffer containing 10% (wt/vol) dextran. After two washings at room temperature for 15 min with 1× SSC/0.1% SDS, the blot was washed twice for 15 min at 42°C with 0.25× SSC/0.1% SDS, dried, and autoradiographed at −70°C for 1–3 days with XAR-5 x-ray film (Eastman Kodak) and an intensifying screen (DuPont).

Scanning Densitometry. Autoradiograms were analyzed by soft laser scanning densitometry (model GS300, Hoefer). Areas under the peaks were determined by digitization on a graphics tablet (Summagraphics, Fairfield, CT) and computer-assisted analysis (R&M Biometrics, Nashville). The areas obtained for c-kit, PDGFRα, and MGSA probes were then corrected for differences in DNA loading as determined with the IL-2 probe. The results for subject B were arbitrarily assigned the value 0.5 for c-kit and PDGFRα probes and 1.0 for MGSA probes. The values for the other subjects were then expressed relative to subject B.

Two-Dimensional β-Particle Counting. To exclude possible nonlinearity of the autoradiograms for the IL-2 control probe, the filter hybridized to the IL-2 probe was also quantitated by two-dimensional β-particle counting (Betascope model 603 blot analyzer). The total counts present in an area of the blot corresponding to each band were determined by computer analysis and corrected by subtraction of background values obtained by averaging the counts in equal blot areas just in front of and behind the band. The areas obtained by scanning densitometry for c-kit, PDGFRα, and MGSA probes were then corrected for differences in DNA loading as determined by β-particle counting of the IL-2 control probe.

Fluorescence *in Situ* Hybridization. Biotin-labeled probes were prepared by nick translation of λ c-*kit* genomic clones G12p and G30p using BIO-11-UTP (Sigma). The conditions

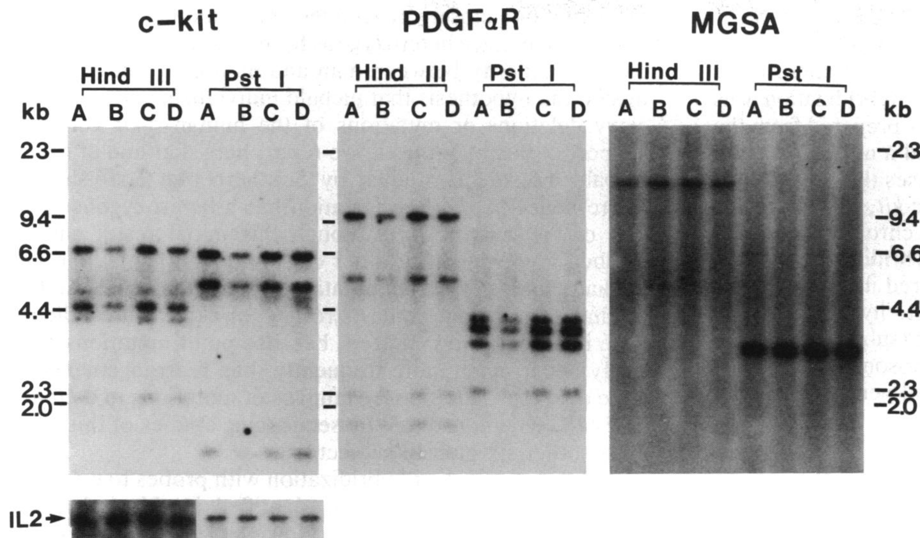

FIG. 2. Hybridization of c-kit, PDGFRα, MGSA, and IL-2 probes to DNAs from four representative individuals with piebald trait. DNAs were prepared, cleaved with *Hin*dIII or *Pst* I, subjected to agarose gel electrophoresis, and transferred to nitrocellulose by Southern blotting. The resulting filter was sequentially hybridized to probes for c-*kit* (*Left*), IL-2 (*Lower Left*), PDGFRα (*Middle*), and MGSA (*Right*) labeled with [^{32}P]dCTP by random priming. Only the major band corresponding to the strongest hybridization with the IL-2 control probe is shown. Size markers in the left and right panels are bacteriophage λ *Hin*dIII fragments.

for fluorescence *in situ* hybridization were carried out as described (22). Briefly, 10 μl of hybridization mixture containing 50% formamide, 10% dextran sulfate, 2× SSC, 1 μg of salmon sperm DNA, 1 μg of human placental DNA, and 0.1 μg of labeled probe DNA was denatured at 70°C for 10 min, preannealed for 15 min at 37°C, and applied to denatured chromosome specimens. After overnight incubation at 37°C and posthybridization washes (22), the biotinylated probe was detected using fluorescein isothiocyanate-conjugated avidin (5 μg/ml, Vector Laboratories). The signal was enhanced by incubation with biotinylated goat-anti-avidin (Vector Laboratories) followed by fluorescein isothiocyanate-conjugated avidin. Chromosomes were counterstained with propidium iodide and diamidinophenylindole.

RESULTS

Phenotypes of Mouse Dominant Spotting and Human Piebald Trait. The pigmentation changes exhibited in piebald trait (Fig. 1*A*) are strikingly similar to those exhibited by mice heterozygous for mutations in c-*kit* (Fig. 1*B*). Although the extent of depigmentation is somewhat variable, all seven human subjects, each from unrelated kindreds, had a congenital white hair forelock and/or patches of hypopigmentation on the abdomen and lower legs.

Southern Blot Analysis of c-*kit*. For initial analysis, genomic DNAs were prepared from lymphoblastoid cell lines derived from peripheral blood lymphocytes. To screen for possible rearrangement or deletion of c-*kit*, a human cDNA probe to the 5′ end of the coding sequence (7) was hybridized to the genomic DNAs after digestion with a variety of restriction enzymes. None of the cases demonstrated rearrangement of the c-*kit* gene (data not shown). However, as seen in the representative digests shown in Fig. 2 *Left*, subject B showed a reduction in band intensity, suggesting the possibility of a heterozygous deletion of c-*kit*.

To control for differences in DNA loading, the blot was stripped and hybridized with a probe for the IL-2 gene (21), which maps to the distal end of chromosome 4q and should be present in diploid copy number in somatic cells. No significant differences in DNA loading were seen with this control probe (Fig. 2 *Lower Left*). Quantitation of the major bands by both scanning densitometry and two-dimensional β-particle counting in a Betascope model 603 blot analyzer confirmed the haploid copy number of c-*kit* in subject B (Table 1). Identical results were obtained with an entirely separate DNA preparation digested with additional different restriction enzymes, excluding errors that might be due to the initial DNA sample preparation or Southern transfer (data not shown).

Southern Blot Analysis of PDGFRα and MGSA. To define the extent of the deletion in subject B, the blot was stripped and hybridized successively to probes for the closely linked genes PDGFRα (14–16) and MGSA (17, 18), which has been proposed as a candidate gene for piebald trait (18). The intensity of the hybridization in subject B is again reduced for PDGFRα (Fig. 2 *Middle*) but not for MGSA (Fig. 2 *Right*). Quantitation by densitometry and β-particle counting confirmed the deletion of PDGFRα but not MGSA (Table 1). Further hybridization with a probe to the transmembrane and kinase domains of c-*kit* revealed a normal pattern of restriction fragments in all subjects, but again with decreased

Table 1. Quantitation of c-*kit*, PDGFRα, and MGSA gene copy number by scanning densitometry and β-particle counting

Restriction endonuclease	Gene copy number ratio											
	kit/IL-2				PDGFRα/IL-2				MGSA/IL-2			
	A	B	C	D	A	B	C	D	A	B	C	D
*Hin*dIII	0.9	0.5	0.9	1.0	1.0	0.5	1.0	1.1	0.8	1.0	1.0	1.1
	(1.0)	*(0.5)*	*(1.1)*	*(0.9)*	*(1.1)*	*(0.5)*	*(1.2)*	*(1.0)*	*(0.9)*	*(1.0)*	*(1.1)*	*(1.1)*
Pst I	1.1	0.5	1.1	1.1	0.9	0.5	1.3	1.1	0.9	1.0	1.2	1.1
	(1.0)	*(0.5)*	*(1.0)*	*(1.1)*	*(0.8)*	*(0.5)*	*(1.2)*	*(1.1)*	*(0.8)*	*(1.0)*	*(1.1)*	*(1.1)*
*Eco*RI	1.2	0.5	1.0	1.0	1.1	0.5	0.9	0.8	1.1	1.0	1.1	0.9
	(1.0)	*(0.5)*	*(0.9)*	*(1.0)*	*(0.9)*	*(0.5)*	*(0.9)*	*(0.8)*	*(0.9)*	*(1.0)*	*(0.8)*	*(0.9)*

Subjects A–D were analyzed. Results for subject B were assigned values of 0.5 for probes c-kit and PDGFRα and of 1.0 for probe MGSA. The values for the three other subjects shown in Fig. 2 are expressed relative to subject B. The lower line of data (in italics) for each subject and restriction digest has been corrected for differences in DNA loading with the IL-2 results obtained by β-particle counting.

intensity in subject B, suggesting that the deletion involves the entire c-*kit* coding region (data not shown).

Fluorescence *in Situ* Hybridization of c-*kit*. To confirm the Southern blot data, fluorescence *in situ* hybridization was performed using metaphase chromosomes prepared from the lymphoblastoid cell lines of subject B and a normal control. The probes, which contained ≈24 kilobases (kb) of genomic sequences from the 3′ end of human c-*kit*, hybridized to 4q11–12, in agreement with the previous chromosome localization (7, 11). As shown in Table 1, hybridization to both copies of chromosome 4 (Fig. 3*a*) occurred in 77 out of 100 normal control metaphases. In contrast, hybridization to metaphases from the piebald trait cell line (subject B) yielded no metaphases with signals on both chromosome 4 homologs, compared to 93 out of 100 with one signal per metaphase at 4q11–12 (Table 2 and Fig. 3*b*).

DISCUSSION

Although the c-*kit* protooncogene is necessary for normal pigmentation in the mouse, its role in human melanocyte development is unclear. In this regard, we have noted that the phenotype of piebald trait, a congenital defect of human pigmentation, closely resembles the "dominant spotting" phenotype of mice heterozygous for mutations in c-*kit*. Based on this similarity between man and mouse, we have investigated the hypothesis that piebald individuals may similarly carry deletions or mutations of the human c-*kit* gene. In support of this hypothesis, we report here that one of seven piebald individuals studied by Southern blot analysis and fluorescence *in situ* hybridization has a heterozygous deletion of the c-*kit* gene. Although this initial result cannot exclude involvement of other closely linked genes, the immediate finding of a c-*kit* mutation, despite the small number of kindreds studied, argues strongly for c-*kit* as a candidate gene in piebald trait. Indeed, because point mutations generally occur much more frequently than rearrangements or large deletions, additional examples of mutations in c-*kit* are likely to be demonstrated by sequencing studies of this gene in the other six piebald subjects.

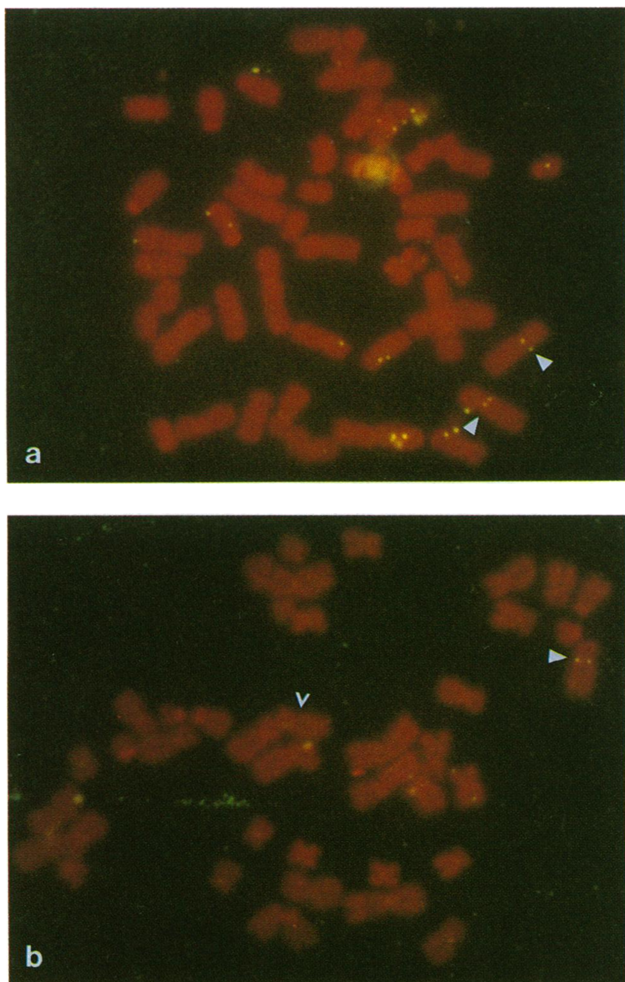

FIG. 3. *In situ* hybridization of genomic c-kit probes to metaphases from a normal control (*a*) and from piebald trait subject B (*b*). The photographs show the hybridization of the biotinylated probes detected with fluorescein isothiocyanate-conjugated avidin. The two solid arrowheads in *a* show probes hybridized to both chromatids in each chromosome 4 at q11–12. The single solid arrowhead in *b* shows probes hybridized to one copy of chromosome 4, and the open arrowhead indicates the other chromosome 4, which shows no signal at q11–12.

Based on the lack of hybridization with probes to c-*kit* and PDGFRα, the deleted region identified in this individual appears to be relatively large. However, by analogy to the closely related gene pair, platelet-derived growth factor receptor β and colony-stimulating factor 1 receptor, which are each 60 kb long but are separated by only 0.5 kb on chromosome 5 (23), a deletion of as little as 100–200 kb may be sufficient to encompass the homologous pair of c-*kit* and PDGFRα on chromosome 4. Indeed, high-resolution banding and cytogenetic analyses of peripheral blood lymphocytes from subject B were normal (data not shown), suggesting that the deleted region is smaller than a single cytogenetic band. Previous cytogenetic studies of large interstitial deletions in a limited number of patients with piebald trait and multiple congenital anomalies have tentatively mapped piebald trait to the proximal end of chromosome 4q (13). The results presented here confirm the cytogenetic mapping and provide molecular evidence mapping piebald trait to the location of c-*kit* within 4q11–12.

The availability of this deletion should facilitate further molecular analysis of this important region of the human genome. Two other mouse loci, Patch (*Ph*) (24) and Rumpwhite (*Rw*) (25), are very closely linked to c-*kit* and also appear to be involved in both pigmentation and embryonic development. Although neither gene has been cloned and their human homologs are unknown, PDGFRα is deleted in *Ph* mutant mice (26), suggesting the possibility that the Ph phenotype is due to a deficiency of this receptor. Mutations in PDGFRα alone appear unlikely to be a cause of piebald trait, however. Although the *Ph* mutation causes spotting in heterozygous mice, it does not produce the characteristic forehead blaze, the mouse counterpart to the white forelock in piebald trait. In addition, a large mouse deletion that involves c-*kit*, *Ph*, and the PDGFRα gene (27), and thus may be analogous to the human deletion described here, results in a dominant spotting phenotype equivalent to that seen with the deletion of c-*kit* alone (28). In accord with the mouse data, the finding of a heterozygous deletion of the human PDGFRα gene, which is thought to play a major role in development

Table 2. Results of *in situ* hybridization in a normal control and subject B with piebald trait

Signals at 4q11–12, no. per metaphase	Metaphases, no.	
	Normal control	Subject B
0	10	7
1	13	93
2	77	0

One hundred metaphases from both a normal control and subject B with piebald trait were analyzed. Only doublet signals (both chromatids) that hybridized to 4q11–12 were counted as positives in the analysis.

(29), does not appear to have had any additional clinical consequence in subject B.

Because mice heterozygous for most mutant alleles of c-*kit* do not manifest the extracutaneous defects seen in homozygous mutants (5), it is not surprising that no association has been reported between piebald trait and anemia or infertility. Indeed, none of the subjects described here was anemic (data not shown). On the other hand, mental retardation and cerebellar signs have been reported in one piebald trait kindred manifesting unusually extensive cutaneous involvement (30). Although homozygous mutant mice have no known neurologic defect, the highest levels of c-kit mRNA in normal tissues are found in the brain, predominantly the hippocampus and cerebellum (31). Moreover, one patient with presumed homozygous piebald trait has been reported (32). This child, both of whose parents were piebald, not only displayed a complete lack of hair and skin pigmentation, consistent with the pigmentation phenotype observed in homozygous mutant mice, but was also severely mentally retarded.

Since heterozygous deletion of a receptor gene would ordinarily result in every cell producing half the normal number of receptors, why are some areas totally depigmented and others normal? One possibility is that the reduction in receptors impairs migration (33), proliferation (34), or survival (35) of the neural crest-derived pigment stem cells, resulting in failure of melanoblast colonization at anatomic sites most distant from the neural crest. Alternatively, melanoblasts migrating into the forehead and other frequently involved areas may be at the low end of a gradient for the ligand of the c-kit receptor (36). If intracellular signaling must exceed a critical threshold for melanoblasts to become activated, a reduction in receptor number could result in failure to reach this threshold in areas with the lowest ligand concentrations.

The present studies implicate a germ-line deletion of c-*kit* as the cause of piebald trait in one kindred. However, alterations in other genes may be responsible for the piebald phenotype in some kindreds. For example, since the c-*kit* gene product is a membrane receptor, one might expect that mutations in the ligand for this receptor could produce a similar defect. In fact, the mouse Steel gene, which causes a similar spotting phenotype in heterozygous mutants, encodes the ligand for c-*kit* (37–40). Thus, in some patients, piebald trait may prove to result from mutations in this gene, rather than in the receptor gene. Now that probes for the ligand gene are available, a direct test of this hypothesis is feasible.

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