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Dominant Negative and Loss of Function Mutations of the c-*kit* (Mast/Stem Cell Growth Factor Receptor) Proto-Oncogene in Human Piebaldism

Richard A. Spritz, Lutz B. Giebel, and Stuart A. Holmes

Departments of Medical Genetics and Pediatrics, University of Wisconsin, Madison

Summary

Piebaldism is an autosomal dominant disorder of melanocyte development and is characterized by congenital white patches of skin and hair from which melanocytes are completely absent. A similar disorder of the mouse, "dominant white spotting" (*W*), results from mutations of the c-*kit* proto-oncogene, which encodes the cellular tyrosine kinase receptor for the mast/stem cell growth factor. We have identified c-*kit* gene mutations in three patients with piebaldism. A missense substitution (Phe→Leu) at codon 584, within the tyrosine kinase domain, is associated with a severe piebald phenotype, whereas two different frameshifts, within codons 561 and 642, are both associated with a variable and relatively mild piebald phenotype. This is consistent with a possible "dominant negative" effect of missense c-*kit* polypeptides on the function of the dimeric receptor.

Introduction

Piebaldism is an autosomal dominant disorder of melanocyte development and is characterized by congenital white patches of skin and hair that completely lack pigment, principally on the forehead, chest and abdomen, and the extremities (Keller 1934; Froggat 1951; Cooke 1952). Melanocytes are completely absent from these regions (Breathnach et al. 1965; Jimbow et al. 1975), and piebaldism is thought to result from defective proliferation or migration of melanocytes from the neural crest during development. Because of its distinctive phenotype, piebaldism has been known since at least ancient Roman times (Lucian 1905) and was one of the first genetic disorders for which a pedigree was presented (Morgan 1786).

A similar disorder of the mouse,"dominant white spotting" (*W*), associated with defects of hematopoiesis and germ-cell development (reviewed in Silvers 1979; Lyon and Searle 1989), has recently been shown to result from mutations of the c-*kit* proto-oncogene (Geisler et al. 1988; Nocka et al. 1990; Reith et al. 1990; Tan et al. 1990; Hayashi et al. 1991), which encodes the tyrosine kinase cell-surface receptor for the mast/stem cell growth factor (Flanagan and Leder 1990; Huang et al. 1990; Williams et al. 1990; Zsebo et al. 1990). The coincident locations of the human c-*kit* gene at 4q11-q12 (Yarden et al. 1987) and of interstitial deletions of chromosome segment 4q12 in some patients with piebaldism (Funderburk and Crandall 1974; Lacassie et al. 1977; Hoo et al. 1986; Yamamoto et al. 1989) initially suggested that human piebaldism might also result from defects of c-*kit,* and we have recently described a c-*kit* gene missense mutation associated with the classic severe piebald phenotype in a large human kindred (Giebel and Spritz 1991). Here we describe three additional c-*kit* gene mutations in unrelated families with piebaldism. A missense substitution (codon 584 Phe→Leu) within the tyrosine kinase domain is associated with the typical severe piebald phenotype. However, two different frameshifts, at codons 561 and 642, both also within the tyrosine kinase domain, are associated with variable but generally milder phenotypes. This difference may relate to "dominant negative" effects that c-*kit*

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Address for correspondence and reprints: Richard A. Spritz, M.D., 317 Laboratory of Genetics, University of Wisconsin, 445 Henry Mall, Madison, WI 53706.

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polypeptides containing amino acid substitutions have on the function of the receptor dimer.

Material and Methods

Description of the Probands

The proband of family 1 (fig. 1, *top*) was an adult Caucasian female with typical piebaldism, including white forelock and extensive nonpigmented patches on the chest, arms, and legs. There was no dysmorphia or heterochromia irides, and hearing was normal. Complete blood counts were repeatedly normal. Her sister, daughter, father, and grandfather were similarly affected. There was no history of dysmorphia, heterochromia irides, deafness, constipation, or anemia in any of the affected individuals; however, both the proband and her sister had experienced some difficulty becoming pregnant.

The proband of family 2 (fig. 1, *middle*) was an adult Caucasian female with typical piebaldism, including white forelock and extensive nonpigmented patches on the arms and legs. There was no dysmorphia or heterochromia irides, and hearing was normal. She reported chronic severe constipation. Complete blood count was normal. Her mother had a white forelock and extensive nonpigmented areas of the abdomen, arms, and legs. The proband's son had extensive nonpigmented areas of the forehead, chest, and arms and experienced very large and infrequent stools. However, her brother had only a very small white forelock and a single small nonpigmented patch on one leg. There was no history of dysmorphic facial features, heterochromia irides, deafness, infertility, or anemia in any of the affected individuals.

The proband of family 3 (fig. 1, *bottom*) was a previously reported (Selmanowitz et al. 1977) adult Caucasian male with mild piebaldism, exhibiting only nonpigmented patches on both legs. There was no dysmorphia or heterochromia irides, and hearing was normal. Complete blood count was normal. His sister was more severely affected, with a white forelock, a nonpigmented patch on the chest, and nonpigmented patches on both legs. All other affected individuals had even milder manifestations: nonpigmented patches limited to a single leg or, in one case, only a white forelock. There was no history of dysmorphic facial features, heterochromia irides, deafness, infertility, or anemia in any of the affected individuals.

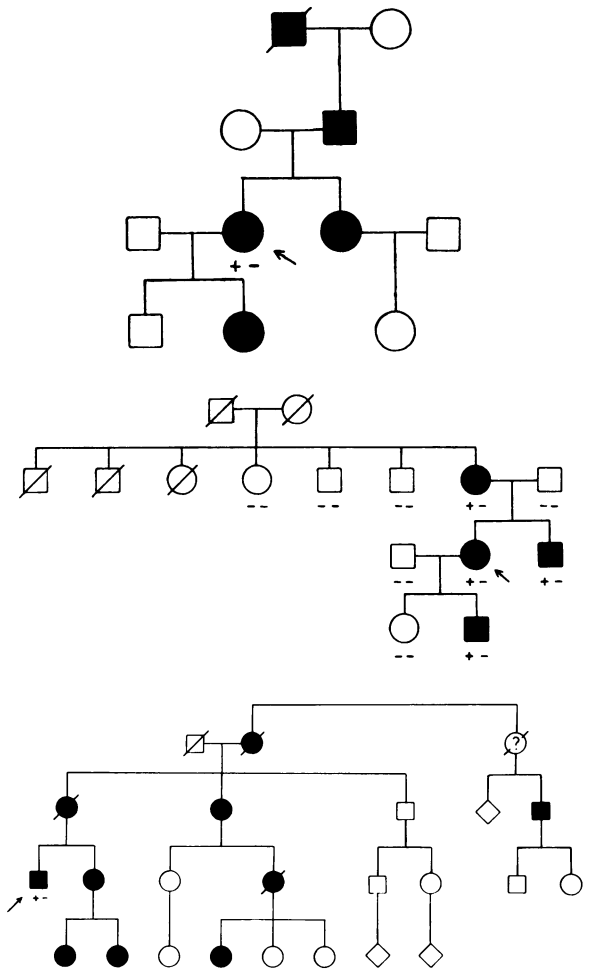

Figure 1 Pedigrees and linkage analysis of piebaldism. *Top,* Family 1. The arrow indicates the proband. Blackened symbols denote individuals with piebaldism, and unblackened symbols denote normal individuals. The plus sign (+) denotes presence of codon 584 mutation, and the minus sign (−) denotes its absence. *Middle,* Family 2. The symbols are as in the top panel, except that + denotes presence of codon 642 frameshift, and − denotes its absence. *Bottom,* Family 3. The symbols are as in the top panel, except that + denotes presence of codon 561 frameshift, and − denotes its absence. The phenotype of the individual indicated by the question mark (?) is not known.

Southern Blot Hybridization

DNA was purified from peripheral blood leukocytes of probands 1 and 2 and of unrelated, normally pigmented individuals, was cleaved with *Xba*I, and was analyzed by blot hybridization (Southern 1975), by using as probes two human c-*kit* cDNAs: phc-*kit*171 (Yarden et al. 1987), containing codons 1–413, and pc-*kit*c427-3′ (Giebel and Spritz 1991), containing codons 427 through the 3′ untranslated region.

PCR Amplification and Sequencing of Genomic DNA

PCR primers designed to amplify each of the 21 c-*kit* coding exons plus adjacent flanking noncoding

Table 1

Oligonucleotide Primer Pairs Used for Amplification of *c-Kit* Gene Exon Segments by PCR

Exon	Primers[a]
1.....	5′-TGGGAGGAGGGGCTGCTG-3′/3′-CCTGACGCCCGGGAGTCA-5′
2.....	5′-ATTGTAGAGTACACAGA-3′/3′-CCTACTGACTCGGTATCTA-5′
3.....	5′-GTGTTTTCAGTGTCTGTGAC-3′/3′-CTGAAGAGAAGAGCAACTAG-5′
4.....	5′-TGTACACATTTGAGGAGAAA-3′/3′-GCTGATCACGCAGACAGTC-5′
5.....	5′-TGGAGAAGTTAATTGCTGCT-3′/3′-CTTCTTTAATAATCTGTCA-5′
6.....	5′-TTGTAATTCCAAGATGAGG-3′/3′-GTCACCCGAATAGTCTAG-5′
7.....	5′-TATGTGTGTGCGTGTTTATG-3′/3′-GTCGACGTTCCTGAGTTGACA-5′
8.....	5′-CTCAGGAAGGTTGTAGGG-3′/3′-AATGACTTACTGAAGAGAT-5′
9.....	5′-TTCCTAGAGTAAGCCAGGG-3′/3′-TGGTATAGTCAGTACTAA-5′
10.....	5′-GATCCCATCCTGCCAAAGTT-3′/3′-CACGAGATTACTGACTCTGTTA-5′
11.....	5′-CAGGTAACCATTTATTTGT-3′/3′-CAAGGTGGACTTTGTTACT-5′
12.....	5′-ATGTCTCTGGACAACATTG-3′/3′-CGGTCAACACGAAAAACGA-5′
13.....	5′-ATCAGTTTGCCAGTTGTGCT-3′/3′-CCGTTACGATCTAATATTT-5′
14.....	5′-GTCTGATCCACTGAAGCTG-3′/3′-CTGTCCGTCAAGTACCCCA-5′
15.....	5′-AACTTTACATGACTTTCCTC-3′/3′-TCAATCCCAACGTTCACCCA-5′
16.....	5′-GAAGTGATCTGCCTGCAAG-3′/3′-TCTTGTCTCGTAAAATCTCGG-5′
17.....	5′-GTTTTCACTCTTTACAAGT-3′/3′-CAAAGGACACTGAAAGTATTACATT-5′
18.....	5′-TGAGCTTCTGAATTAACAT-3′/3′-TGTTCTACTAGTTCCTTCC-5′
19.....	5′-GATCCTTGCCAAAGACAAC-3′/3′-AGAAATACTGTGTGACCAG-5′
20.....	5′-TACTGAAGTTGCTGGATGC-3′/3′-GGGTCAAGGTCCACACAGG-5′
21.....	5′-AGTATGCCTTTTGTTGCTAT-3′/3′-CACGACAGTACACCCACTT-5′

[a] Derived from intervening and flanking sequences, thus amplifing exons plus considerable adjacent noncoding sequences.

sequences were derived from the normal human c-*kit* genomic DNA sequence (authors' unpublished data). Each exon was amplified from genomic DNA (0.1 μg) of the probands by 35 cycles of PCR (Saiki et al. 1988) by using paired oligonucleotide primers (table 1), *Taq* DNA polymerase (AmpiTaq; Perkin Elmer—Cetus), and an automated thermal cycler (Coy Laboratory Products). Each cycle consisted of 30 s at 94°C, 1 min at 53°C, and 1 min/kb at 72°C. The products of two replicate PCR amplifications were pooled and were purified by electrophoresis through 4% polyacrylamide gels. The bands were eluted from the gels, were extracted once with phenol-chloroform, and were precipitated by ethanol. For probands 1 and 2, coding exons 2, 4, 8, 13, 14, and 21, the complete DNA sequences of the purified, double-stranded PCR products were determined directly, essentially by the method of Sanger et al. (1977) by using α-[^{35}S]-dATP and *Taq* DNA polymerase. For coding exons 1, 3, 5–7, 9, 10–12, and 15–20, for which direct DNA sequence analysis was not technically satisfactory, the PCR products were cloned into M13mp19, and the nucleotide sequences were determined for at least six independent clones by using single-stranded DNA templates. For proband 3, the PCR products of all 21 exons were cloned into M13mp19 for DNA sequence analysis. DNA sequences were analyzed using the DNAStar software package.

Allele-specific Oligonucleotide Hybridizations and Genetic Linkage Analyses

For analyses of the c-*kit* codon 584 mutation, 328-bp DNA segments containing c-*kit* exon 11 were amplified from genomic DNAs (0.1 μg) of the proband and of 55 unrelated, normally pigmented Caucasian individuals by the PCR as described above. One-fifth (20 μl) of each reaction was applied to ZetaProbe nylon membranes (Bio-Rad) with a Bio-Dot SF microfiltration apparatus (Bio-Rad). Allele-specific oligonucleotide hybridizations of replicate filters were carried out according to a method described elsewhere (Kogan and Gitschier 1990), by using 5′-end-radiolabeled 19-mer oligonucleotide probes corresponding to the normal (5′-ATGGGAGTTTCCCAGAAAC-3′) and mutant (5′-ATGGGAGTTGCCCAGAAAC-3′) forms of codon 584.

For analyses of the c-*kit* codon 642 mutation, 250-bp DNA segments containing c-*kit* exon 13 were PCR

amplified from genomic DNAs of the proband, all living family members, and 20 unrelated, normally pigmented Caucasian individuals as described above. Allele-specific oligonucleotide hybridization analyses were carried out as above by using 5′-end-radiolabeled 19-mer oligonucleotide probes corresponding to the normal (5′-CTGAACTCAAAGTCCTGAG-3′) and mutant (5′-TCTGAACTCAGTCCTGAGT-3′) forms of codon 642. Genetic linkage of the codon 642 mutation to the piebald phenotype was assessed by determination of the logarithm of odds (lod) scores (Z) by using version 3 (1987) of the LIPED program (Ott 1974).

Results

The Proband of Family 1 Is Heterozygous for a Missense Mutation at Codon 584 (Phe→Leu)

Preliminary Southern blot analysis of DNA of the proband of family 1, by using c-*kit* cDNA fragments as probes, identified no c-*kit* gene deletion or other rearrangement (data not shown). Therefore, all 21 c-*kit* coding exons plus adjacent portions of the intervening and 5′ and 3′ untranslated and flanking sequences were amplified by PCR and were sequenced from DNA of the proband. This analysis, of 8.3 kb of DNA, demonstrated only a single-base difference from the normal gene, a TTT→TTG substitution in codon 584, within exon 11 (fig. 2). This results in a phenylalanine-to-leucine substitution in the tyrosine kinase domain of c-*kit* (Yarden et al. 1987). As expected for an autosomal dominant disorder, the proband was heterozygous for this change.

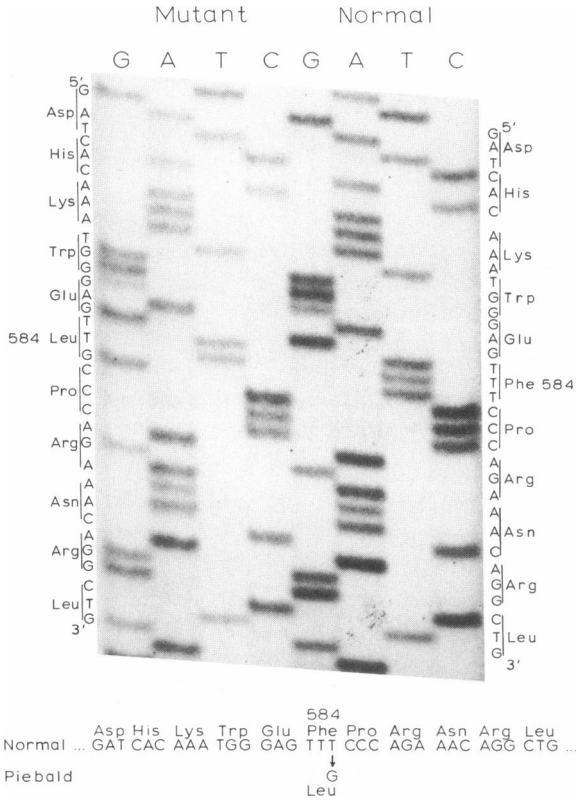

Figure 2 Sequences in region of codon 584 c-*kit* gene mutation from two alleles of proband of family 1. The sequence shown is that of the coding strand. Note that the sequence of exon 11 was determined by cloning in M13 and that each template thus illustrates only one allele.

The Proband of Family 2 is Heterozygous for a Frameshift at Codon 642

Preliminary Southern blot analysis of DNA of the proband of family 2, by using c-*kit* cDNA fragments as probes, also identified no c-*kit* gene deletion or other rearrangement (data not shown). DNA sequence analysis of her PCR-amplified c-*kit* exon segments, as described above, demonstrated only a single difference from normal, a two-base deletion in codon 642 (AAA→A– –), within exon 13 (fig. 3), also within the tyrosine kinase domain. This results in a frameshift distal to codon 641, terminating only six residues downstream at a novel in-frame TAA. As expected, the proband was heterozygous for this change.

The Proband of Family 3 Is Heterozygous for a Frameshift at Codon 561

DNA sequence analysis of the PCR-amplified c-*kit* exon segments of the proband of family 3 also demonstrated only a single difference from normal, a one-base duplication in codon 561 (GAG→GGAG), within exon 11 (fig. 4), also within the tyrosine kinase domain. This results in a frameshift distal to codon 560, terminating 18 residues downstream at a novel in-frame TGA. As expected, the proband was heterozygous for this change.

The Codon 584 and Codon 642 Mutations Are Not Common Polymorphisms

To determine whether the codon 584 and codon 642 mutations represent nonpathological polymorphisms, we analyzed the PCR-amplified exon 11 and exon 13 fragments from the probands of families 1 and 2 and from a number of unrelated, normally pigmented Caucasians, by allele-specific hybridization using oligonucleotides that corresponded to the nor-

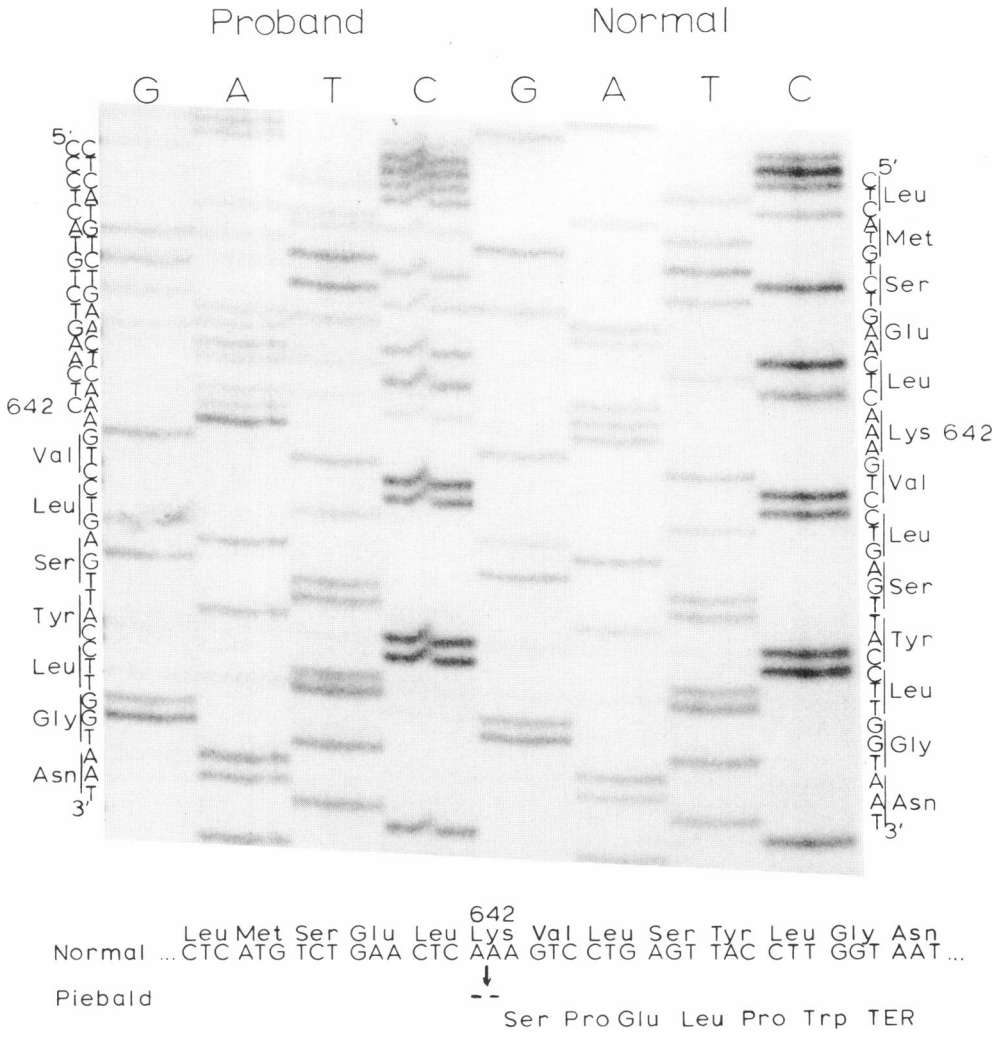

Figure 3 Sequences in region of codon 642 c-*kit* gene frameshift from proband of family 2 and unrelated normal person. The sequence shown is that of the coding strand. Note that the sequence of exon 13 was determined directly, without cloning, and thus sampled both alleles simultaneously.

mal and mutant forms of codons 584 and 642. The proband of family 1 was heterozygous for the codon 584 mutation, whereas 55 controls had only the normal codon 584; none had the mutant form (data not shown). The proband of family 2 was heterozygous for the codon 642 mutation, whereas 20 controls had only the normal codon 642; none had the mutant form (data not shown). In addition, DNA sequence analyses of three additional unrelated probands with piebaldism showed that none had the codon 584 and codon 642 mutations (Giebel and Spritz 1991; authors' unpublished data). Therefore, the codon 584 and codon 642 mutations are not common nonpathological polymorphisms.

The Codon 642 Mutation Is Associated with the Piebald Phenotype in Family 2

In families 1 and 3, only the probands were studied; other family members were not available for analysis. However, all living members of family 2 were available for study. To determine whether the codon 642 frameshift is genetically linked to piebaldism in this family, we carried out allele-specific oligonucleotide hybridization analysis of the codon 642 mutation in

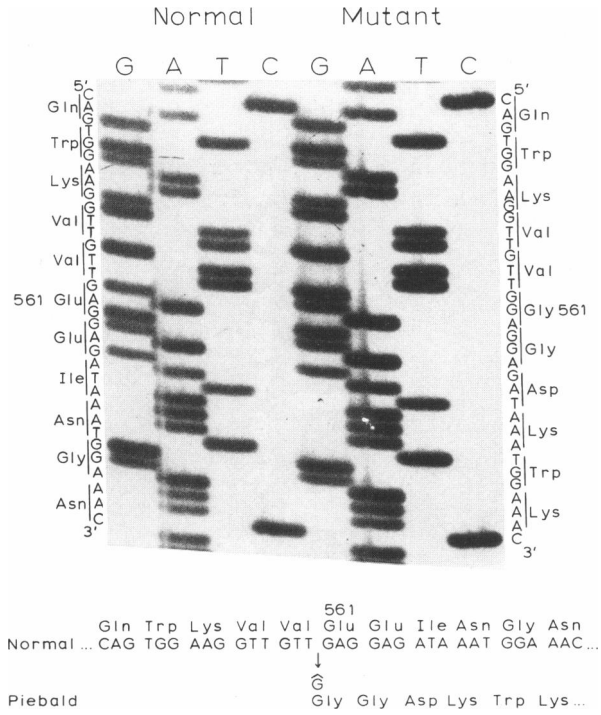

Figure 4 Sequences in region of codon 561 c-*kit* gene frameshift from proband of family 3 and unrelated normal person. The sequence shown is that of the coding strand. Note that the sequence of exon 11 was determined by cloning in M13 and that each template thus illustrates only one allele.

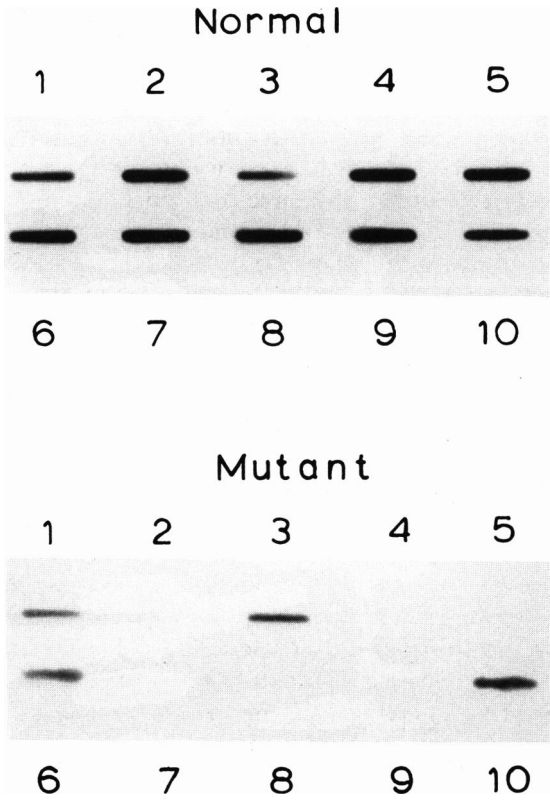

Figure 5 Allele-specific hybridization of codon 642 frameshift in family 2. Oligonucleotides corresponding to the normal and mutant versions of codon 642 were hybridized to exon 13 PCR products from all living relatives of the proband. 1 = son; 2 = daughter; 3 = proband; 4 = husband; 5 = father; 6 = mother; 7 = uncle; 8 = uncle; 9 = aunt; and 10 = brother.

exon 13 segments PCR amplified from genomic DNA of all family members, as described above. As shown in figure 5, there was perfect concordance between heterozygosity for the codon 642 mutant allele and the piebald phenotype in this family; all affected family members had both the normal and mutant allele, and all normal individuals had only the normal allele. Two-point linkage analysis, performed with the LIPED program (Ott 1974), yielded a maximal Z (Z_{max}) of 1.81 at recombination fraction (θ) = 0. This is consistent with, although it does not prove, genetic linkage of the codon 642 frameshift to the piebald phenotype in this family.

Discussion

Piebaldism is a rare autosomal dominant disorder characterized by congenital patches of white skin and white hair. The white patches, which may be surrounded by regions of increased pigmentation, occur most frequently on the face, chest, abdomen, and extremities. The nonpigmented patches entirely lack melanocytes (Breathnach et al. 1965; Jimbow et al. 1975), apparently because of defective proliferation or migration of melanoblasts from the neural crest to the epidermis during embryogenesis. Patients with piebaldism (Mahakrishnan and Srinivasan 1980) and other "white spotting" disorders such as Waardenburg syndrome occasionally also lack ganglion cells of the intestinal enteric neural plexus, which are also derived embryologically from the neural crest. Mice with "dominant white spotting" (*W*), the murine homologue to human piebaldism, may also manifest defects of hematopoiesis and germ-cell development (reviewed in Silvers 1979; Lyons and Searle 1989), although these have never been reported in piebald humans.

We have previously identified a missense substitution of the c-*kit* gene, codon 664Gly→Arg, in a large

kindred with classic autosomal dominant piebaldism (Giebel and Spritz 1991). That mutation was not detected among any normally pigmented individuals but was tightly linked to the occurrence of a uniformly relatively severe piebald phenotype in that kindred (Z_{max} = 6.02; θ = 0). Here we report three novel c-*kit* gene mutations in three additional families with piebaldism. In family 1 we found a missense substitution at codon 584 (Phe→Leu). In family 2 we found a frameshift at codon 642. In family 3 we found a frameshift at codon 561. These mutations were not observed among normally pigmented individuals, and the aggregate Z linking c-*kit* gene mutations to piebaldism is 7.83 at θ = 0. Moreover, the nature of the mutations observed, especially the codon 561 and codon 642 frameshifts, strongly supports the hypothesis that these mutations cause piebaldism in these families. In addition, the two known human c-*kit* missense substitutions—the codon 584Phe→Leu substitution described here and the codon 664Gly→Arg substitution we described elsewhere (Giebel and Spritz 1991)—closely parallel two of the four known c-*kit* substitutions in *W* mice. The codon 584 human c-*kit* substitution is only one amino acid away from the codon 582Glu→Lys substitution in W^{37} mice (Nocka et al. 1990; Reith et al. 1990; note that the codon numbering of the human and murine c-*kit* polypeptides is slightly different). This region is just upstream from the first part of the ATP-binding site, and substitutions in this region may thus interfere with either ATP binding or hydrolysis. The codon 664 human substitution is only four amino acids away from the codon 660Thr→Met substitutions in W^{v} and W^{55} mice (see above; the *W* and W^{55} alleles are identical). The phenotype associated with heterozygosity both for the human codon 584 and codon 644 mutant alleles and for the murine W^{37} allele is one of white spotting but no anemia. The heterozygous phenotype of the W^{v}/W^{55} allele is similar but includes mild anemia. Homozygosity for the murine W^{37} allele results in both severe anemia and postimplantation lethality, whereas murine W^{v}/W^{55} homozygotes are viable, sterile black-eyed whites with severe anemia. The only reported human with homozygous piebaldism had no pigment of the skin or hair, blue irides, facial dysmorphia, deafness, developmental delay, but no anemia (Hultén et al. 1987); unfortunately, this patient is now deceased.

The codon 584 and codon 664 substitutions of human c-*kit*, as well as the four known missense substitutions of murine c-*kit* (Nocka et al. 1990; Reith et al. 1990; Tan et al. 1990), all occur within the intracellular tyrosine kinase domain. It seems likely that mutations in the extracellular ligand-binding domain, which consists of five immunoglobulin-like repeats (Yarden et al. 1987), may be functionally silent because of functional redundancy in this region. The biological action of the c-*kit* receptor tyrosine kinase probably requires dimerization in response to ligand binding (Tan et al. 1990; reviewed in Pawson and Bernstein 1990). Heterodimeric receptors containing one normal and one qualitatively abnormal c-*kit* polypeptide may be functionally impaired, resulting in as much as 75% decreased cellular response to ligand binding in individuals heterozygous for c-*kit* alleles containing missense substitutions in the tyrosine kinase domain. However, the amount of functional receptor dimer may be decreased by only 50% or even less in individuals heterozygous for c-*kit* alleles associated with quantitative decreases of polypeptide; such alleles might contain deletions, nonsense and frameshift mutations, and mutations affecting transcription, pre-mRNA splicing, and polyadenylation. This is consistent with the uniformly relatively severe piebald phenotype in the families with the codon 664 (Giebel and Spritz 1991) and codon 584 (family 1) substitutions, versus the quite variable but generally milder phenotype in families 2 and 3, which have the codon 642 and codon 561 frameshifts, respectively. Similar phenomena have been reported in mice with different *W* mutant alleles (Nocka et al. 1990; Reith et al. 1990). Missense mutations of c-*kit* thus behave like "antimorphs" (Muller 1932) or "dominant negative mutations" (Herskowitz 1987), whereby the mutant allele dominantly interferes with function of the wild-type allele.

Piebaldism, its murine homologue "dominant white spotting" (*W*), and the complementary murine defect *Steel* are thus paradigmatic of a new class of genetic disorders involving defective development of specific cell lineages. It is likely that many other genetic human disorders involving specific developmental lineages—disorders including Waardenburg syndrome, Black-fan-Diamond anemia, and cartilage-hair hypoplasia, to name but a few—will ultimately be found to involve similar types of pathogenetic mechanisms.

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