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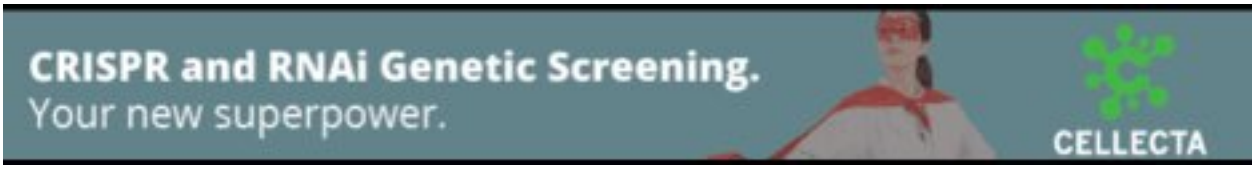
Genome Res. 1995 5: 29-41
Access the most recent version at doi:10.1101/gr.5.1.29



RESEARCH

Quantitative Trait Loci That Modify the Severity of Spotting in *piebald* Mice

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Mice homozygous for the recessive mutation *piebald* (*s*) exhibit a white-spotted coat caused by the defective development of neural crest-derived melanocytes. The severity of white spotting varies greatly, depending on the genetic background on which *s* is expressed. A backcross between two inbred strains of *s/s* mice that exhibit large differences in the degree of spotting was used to identify six genetic modifiers of *piebald* spotting on chromosomes 2, 5, 7, 8, 10, and 13. The loci differed in their spatial contribution to spotting on the dorsal versus ventral surfaces of mice; nonadditive interactions were observed between loci on chromosomes 2 and 5. This study underscores the power of using genetic analyses to identify and analyze loci involved in modifying the severity of phenotypic traits in mice.

A classic approach to the identification of interacting genes in a pathway has been to screen for suppressors and enhancers of a phenotypic trait. Although such mutagenesis screens are theoretically feasible in mice, the large genome size, coupled with the relative inefficiency of mutagens, requires that large numbers of animals be generated (Rinchik and Russell 1990; Rinchik et al. 1990). An alternative approach to identifying genes that are involved in a specific phenotype is to identify loci that modify its severity using naturally occurring variations in existing inbred strains (Hilbert et al. 1991; Rise et al. 1991; Todd et al. 1991; Jacob et al. 1992; Dietrich et al. 1993; Ghosh et al. 1993; Agui et al. 1994; Berrettini et al. 1994). Because the genetic contributions to these traits are often caused by a combination of effects at multiple loci as well as epigenetic factors, these traits are termed complex or quantitative genetic traits.

One example of a quantitative trait is the severity of white spotting observed in *piebald* (*s*) mice. Mice homozygous for this recessive mutation exhibit a pigmented and white-spotted coat caused by a lack of the melanin–producing melanocytes in the white areas (Silvers 1956). The work of Dunn and Charles in the 1930s demonstrated clearly that the severity of white spotting in *piebald* mice was dependent on the contribution of multiple genes having quantitative effects (Dunn 1937; Dunn and Charles 1937). By selectively breeding for *s/s* mice that exhibited large variations in the severity of white spotting, a set of modifiers was described, termed the *k–complex*. Although the genetic studies of Dunn and Charles were extensive, it was not technically possible at the time to map or identify the genes comprising the *k-complex* of modifiers.

The analysis of complex genetic traits, such as *piebald* spotting, requires a comprehensive genetic map. By measuring the frequency with which a set of genetic markers is inherited with the phenotype of interest, linkage can be established to the loci involved in modifying that phenotype. With the advent of simple sequence repeat DNA markers that are distributed throughout the entire genome (Dietrich et al. 1992, 1993, 1994), it is now possible to rapidly map the loci that contribute to complex genetic traits. This study describes our efforts to localize *k-complex* modifiers in the mouse genome and demonstrates the power of this approach to analyze gene interactions involved in complex genetic traits in experimental organisms.

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RESULTS

Phenotype Analysis of *piebald* Spotting

In an effort to identify genes involved in melanocyte development, we set out to map loci that are responsible for modifying the severity of white spotting in *piebald* mice. The two strains of mice used in this study display different amounts of white spotting on their coats (Fig. 1). The C3H *s/s* mice have a white patch of hair on the head and abdomen with very few white patches on the dorsal surface, whereas the Mayer strain of *s/s* mice exhibits extensive white spotting on both the dorsal and ventral surfaces.

A cross between these two strains of mice yielded F_1 mice that exhibited a low degree of spotting, indistinguishable from the C3H *s/s* strain. Male and female F_1 *s/s* animals were backcrossed to the Mayer *s/s* mice, and 291 backcross (BC_1) offspring were generated. For ventral spotting, the BC_1 animals were normally distributed with few animals exhibiting the phenotypes of the extreme and the majority of animals exhibiting intermediate phenotypes (Fig. 2). This distribution is characteristic of a trait that is caused by multiple, additive loci. In contrast, the dorsal spotting in these animals was skewed toward low spotting indicating that genetic determinants may have different effects on the dorsal and ventral surfaces. Alternatively, a threshold may exist for dorsal spotting, such that BC_1 animals with less than the minimal number of modifiers do not overcome this threshold and thus do not exhibit an increase in spotting on the dorsal surface. As expected, the total spotting, the average of the dorsal and ventral pattern of each animal, demonstrated a fairly normal-shaped distribution that is slightly skewed because of the contribution of dorsal spotting. Examination of a scatter plot comparing the correlation between dorsal and ventral spotting reveals a general trend whereby dorsal and ventral spotting are correlated; however, a wide range of scatter was observed ($r = 0.635$).

From the distribution of spotting in the BC_1 animals, a lower estimate of three loci that contribute to spotting in Mayer *s/s* mice was calculated (Table 1). Because this formula relies on a normal-shaped distribution and an equal and additive contribution from each locus, it is likely to be a minimum estimate (Wright 1952; Lander and Botstein 1989).

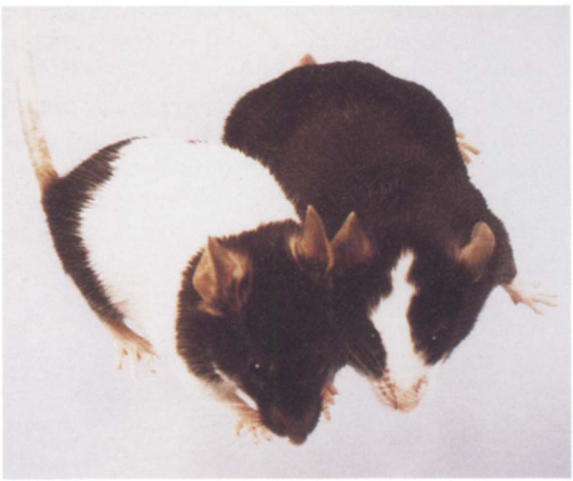

Figure 1 The two strains of *s/s* mice used in this study. The Mayer *s/s* mouse (*left*) exhibits a high degree of spotting with a sharp delineation between the pigmented and nonpigmented areas of the coat. The C3H *s/s* mouse (*right*) exhibits a reduced amount of spotting on both dorsal and ventral surfaces.

Linkage Analysis

To identify the regions of the genome that contain modifying loci, 21 of the whitest animals of the first 150 BC_1 animals were genotyped using 70 microsatellite markers distributed at ~30-cM intervals throughout the mouse genome (Table 2; Fig. 3). Markers were chosen based on three criteria: (1) that together, they provide a 20-cM sweep radius of the genome; (2) that they were polymorphic between the two *s/s* strains; and (3) that they were detectable without employing the use of radioisotopes. Markers that were homozygous for the Mayer *s/s* allele in at least 15 of those 21 animals were considered good candidates for loci that modify the severity of white spotting ($P < 0.05$). From this analysis, six loci that showed potential linkage to increased white spotting were identified on chromosomes 2, 5, 7, 8, 10, and 13 (Fig. 4).

As a second-level screen, the six positive markers, along with additional microsatellite DNA markers surrounding them, were typed for homozygosity in the 60 whitest animals and for heterozygosity in the 30 darkest of the 291 BC_1 animals (Fig. 5). Linkage was tested at each locus using a X^2 analysis with the null hypothesis that homozygosity for a marker is not linked to high spotting and heterozygosity for a marker is not linked to low spotting. Rejection of this null hy-

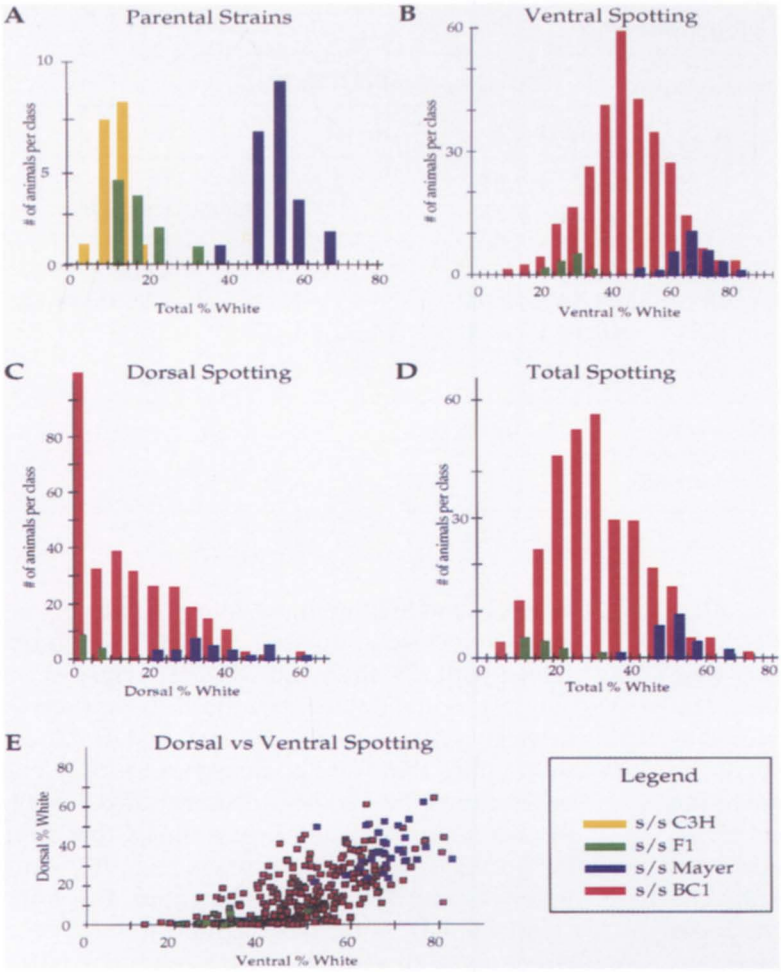

Figure 2 Distribution of white spotting in the parental *s/s* strains, F_1, and backcross progeny. The severity of white spotting on the surfaces of mice was expressed as a percentage of the surface lacking pigmented hair. These data were placed into 5% increments of spotting and plotted against the number of animals in each class. (*A*) Spotting in C3H *s/s* and Mayer *s/s* parental strains and F_1 hybrids. (*B–D*) Distribution of spotting in 291 backcross progeny on the ventral, dorsal, and total surfaces, respectively. (*E*) Correlation analysis of spotting on the dorsal and ventral surfaces in backcross progeny (correlation coefficient, $r = 0.635$).

pothesis implicates linkage of the marker to increased spotting. The critical value (calculated using the X^2 equation with one degree of freedom) for each marker tested is plotted relative to the recombination distance between markers (Fig. 5). All six loci maintained their linkage to total spotting ($P < 0.05$), although in several instances the inclusion of surrounding markers refined the map position of the modifier. For example, D5Mit23, D8Mit47, and D13Mit8 showed tighter linkage to the modifier than the original markers used.

The markers D10Mit12 and D8Mit47 exhibited significant linkage ($P < 0.001$), irrespective of whether the calculation was performed with the whitest or darkest mice alone or in combination, suggesting that homozygosity at these loci contributes to white spotting and heterozygosity contributes to the lack of spotting. In contrast, for D2Mit1 and D7Mit57, the significance of linkage declined when the darkest animals were included, suggesting these loci contribute to increased spotting when homozygous for the Mayer allele but only in combination with other loci (see below).

While this second-level screen confirmed the likelihood of linkage to the six loci identified in the initial screen, both analyses utilized only those animals at the extremes of the phenotype spectrum and therefore were biased by any variations in that subgroup. For a more stringent determination of linkage, the markers that exhibited the highest critical values in the second-level screen were typed in all 291 BC_1 animals. Linkage was assessed using a Mann–Whitney U test (Table 2) which compares the ranks of spotting values between the heterozygotes and homozygotes rather than comparing the actual percentage of spotting. This test does not require a normal distribution of the phenotypes and is not greatly influenced by extremes in the sample population. Loci on chromosomes 2, 5, 8, and 10 demonstrated a strong correlation with linkage ($P < 0.001$), whereas the loci on chromosomes 7 and 13 resulted in weaker correlations ($P < 0.02$ and $P < 0.06$, respectively), consistent with the findings in Figure 5.

The Mann–Whitney U test analysis of the six linked marker loci did not allow us to determine the map position of the modifier loci relative to the linked markers. Relative order was determined by genotyping all 291 BC progeny with additional linked markers and determining the probability that the modifier was located between two adjacent markers (Fig. 6). The recombinant animals were scored as being homozygous

Table 1. Phenotypes of *s/s* strains and progeny of crosses

Strain or cross	N[a]	Spotting (%)[b] ventral	dorsal	total
s/s C3H	17	25.77 ± 5.61	2.89 ± 1.21	14.96 ± 3.25
s/s Mayer	28	67.73 ± 6.78	38.17 ± 10.35	53.50 ± 6.24
(*s/s* Mayer × *s/s* C3H)F_1	12	31.26 ± 7.88	2.99 ± 3.01	17.30 ± 5.85
s/s BC_1 total	291	48.96 ± 12.24	15.22 ± 13.37	32.09 ± 11.57
(*s/s* Mayer × *s/s* C3H)F_1 × *s/s* Mayer	182	49.44 ± 12.66	15.95 ± 13.58	32.69 ± 11.88
s/s Mayer × (*s/s* Mayer × *ss* C3H)F_1	109	48.18 ± 11.51	14.00 ± 12.98	31.09 ± 11.02
Estimate of number of modifiers[c]		3.8	1.9	3.3

[a](*N*) The number of animals analyzed in each strain or cross.
[b]Spotting is represented as mean percent white on the body surface ± the S.D.
[c]Determined using the formula of Wright (1952; see Methods).

or heterozygous for the Mayer modifier allele based on the severity of total spotting. Recombinant progeny in the whitest 125 animals were scored as homozygous for the Mayer allele, and those in the darkest 125 animals were scored as heterozygous for the Mayer allele. Results of the Mann–Whitney U test are plotted relative to the recombination distance along the chromosome. The likelihood that a modifier locus was between two markers was determined by comparing the difference in probability (*P* value) of the locus residing in each interval (Fig. 6). The modifiers on chromosomes 5, 7, 8, 10, and 13 (indicated by M) are at least one order of magnitude more likely to reside at the indicated positions than at adjacent intervals. Repetition of this analysis using only the recombinant animals resulted in similar conclusions.

Genetic Interactions among Modifier Loci

The data in Figure 5 and Table 3 suggest that the six *piebald* modifiers are not equivalent in their effects on spotting, either in terms of the magnitude of their effects or on their ability to act alone. To assess the possibility that nonadditive interactions occur among the modifier loci, a six-factor ANOVA was performed using the 291 BC_1 animals typed with the marker demonstrating the strongest linkage to each modifier. If two loci act independently and additively, then the degree of spotting will be lowest in those animals that are heterozygous for both loci (open squares in Fig. 7), intermediate in the animals that are homozygous for one of the pair (half-filled squares), and highest in the animals homozygous for both loci (solid squares). Such an effect is observed with D5Mit23 and D8Mit47 (Fig. 7A). In contrast, nonadditive interactions were observed between only two loci, D2Mit1 and D5Mit23, where mice that were homozygous for only one but not the other modifier demonstrated very little increase in spotting over animals that were heterozygous for both modifiers (Fig. 7B). Only when animals were homozygous for both D2Mit1 and D5Mit23 did a significant increase in either dorsal or ventral spotting result ($P < 0.01$). Neither D2Mit1 or D5Mit23 demonstrated this effect when in combination with any other modifier locus. This finding suggests that the modifiers defined by D2Mit1 and D5Mit23 must function in concert as recessive alleles to affect *piebald* spotting.

The Effects of *piebald* Modifiers on Dorsal and Ventral Spotting

To determine whether the modifier loci increased spotting in a spatially restricted pattern, the effect of each locus on dorsal versus ventral surfaces was analyzed (Table 4). To determine the effects on the dorsal relative to the ventral surfaces, the absolute values of percent spotting were converted to increase in percent spotting, a value that can be compared between the two surfaces. Increase in percent spotting on each surface of each mouse homozygous for a given marker was calculated as the difference from the mean of animals heterozygous for that marker. The distributions of these values obtained for the

Table 2. Microsatellite markers used for this study

Marker	cM[a]	Rx[b]	Marker	cM[a]	Rx[b]	Marker	cM[a]	Rx[b]	Marker	cM[a]	Rx[b]
D1Mit1	4.7	1	D6Mit1	3.5	2	D10NDS1	2.3	1	D15Mit5	18	2
D1Mit10	57	1	D6Mit29	28	2	D10Mit3	14.5	1	D15Mit31	36	2
D1Mit14	83	1	D6Mit36	39.7	2	D10Mit10	49.6	1	D15Mit42	58	2
D1Mit17	112	1	D6Mit25	50.5	2	D10Mit12	51.8	1		63.6*	
	118*		D6Mit14	60.8	1	D10Mit103	74.5	2	D16Mit9	3.9	2
D2Mit1	0	1		64.4*			74.5*		D16Mit4	26.9	1
D2Mit115	1.1	2	D7Mit21	2.5	2	D11Mit2	2.2	1		55*	
D2Mit7	26	1	D7Mit75	3.4	2	D11Mit20	18	2	D17Mit21	10	1
D2Mit13	49.5	1	D7Mit56	4.5	2	D11Mit38	44	2	D17Mit7	28.4	1
D2Mit30	56.2	1	D7Mit57	6.7	2	D11Mit14	61	1	D17Mit38	44.9	2
D2Mit51	79	2	D7Mit55	12.3	2	D11Mit12	75.5	1		52*	
	97*		D7Mit54	13.4	2		83.6*		D18Mit14	10.2	2
D3Mit3	16.7	1	D7NDS1	29.3	1	D12Mit35	22.3	2	D18Mit9	27.6	1
D3Mit10	38.3	1	D7Mit40	43.9	2	D12Mit4	30	2	D18Mit7	36.1	2
D3Mit17	51.6	1	D7NDS4	70.5	1	D12Mit8	57.4	2		45.3*	
D3Mit45	59.5	2		70.9*			60*		D19Mit16	13.8	2
	68.6*		D8Mit4	7.9	1	D13Mit17	2.2	2	D19Mit11	27.3	2
D4Mit39	10.3	2	D8Mit8	30.6	3	D13Mit64	14.5	2		59*	
D4Mit9	31.5	1	D8Mit57	38.9	2	D13Mit8	24.4	2	DXMit16	38.8	2
D4Mit12	47.5	1	D8Mit11	41.7	1	D13Mit11	24.4	2		73*	
D4Mit13	62.2	1	D8Mit47	50.7	2	D13Mit9	25.5	1			
	73.4*		D8Mit42	68.2	2	D13Mit37	35.6	2			
D5Mit13	15.8	2		70.9*			61*				
D5Mit15	27.7	2	D9Mit4	22.5	1	D14Mit14	8.9	2			
D5Mit23	38.4	2	D9Mit11	41.7	1	D14Mit39	31.3	2			
D5Mit26	47.4	2	D9Mit12	50.2	1	D14Mit35	46.4	2			
	87*		D9Mit19	65	1		64.8*				
				69.7*							

[a]Chromosome positions are extracted from MIT genome data base (Dietrich et al., 1992). The centimorgan (cM) distances are inferred relative to the most proximal (0 cM) and distal (*) molecular markers typed on each chromosome.
[b]PCR reaction cycling conditions (see Materials and Methods): (1) 94°C for 20 sec, 54°C for 20 sec, and 72°C for 40 sec; (2) 94°C for 30 sec, 54°C for 30 sec, and 72°C for 45 sec; and (3) 94°C for 30 sec, 54°C for 30 sec, and 72°C for 30 sec.

dorsal and ventral surface of each mouse were then compared using a Wilcoxon Signed Rank test and a Paired Sign test (Table 4). Of the six modifier loci analyzed in this way, only the locus mapping to chromosome 10 demonstrated a significantly greater influence on spotting on the dorsal as opposed to the ventral surfaces of *piebald* mice.

DISCUSSION

Quantitative trait loci (QTL) analysis (Lander and Botstein 1989) was employed to identify the six loci that modify the severity of spotting in two strains of *piebald* mice that exhibit vastly different amounts of both dorsal and ventral spotting. The gene for *piebald* has been identified recently by Hosada and colleagues (1994), who showed that a targeted disruption in the endothelin-B receptor (EDNRB) gene failed to complement a severe allele of *piebald*, known as *piebald lethal*. The endothelins are a group of three small peptide ligands that were originally identified as powerful vasoconstrictors in mammals (Warner 1993). They act through two different cell surface receptors of the G-protein-coupled, seven-transmembrane receptor family, one of which is the *piebald* gene product. The mice used in this study carry the hypomorphic *s* allele of *piebald* that expresses ~25% of wild-type levels of EDNRB mRNA in adult tissues of 129/J mice, most probably caused by a defect in RNA processing or stability (Hosada et al. 1994). Although the *s* allele in the Mayer *s/s* strain was presumed to be iden-

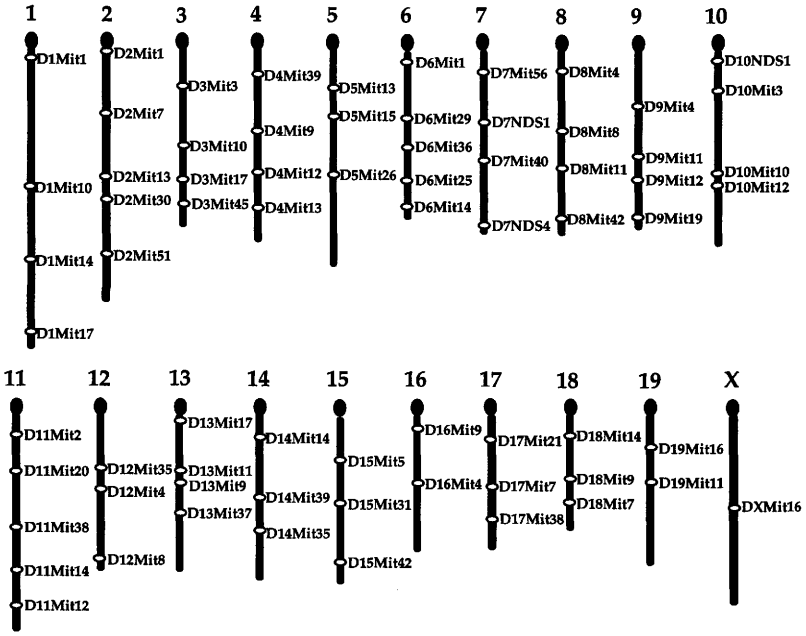

Figure 3 Distribution of microsatellite markers in the first level screen. The lengths of the 19 autosomes and the X chromosome (solid lines) and the positions of the markers (white ovals) were based on recombination distances provided by the MIT mouse genome data base (Dietrich et al., 1992; see Table 2).

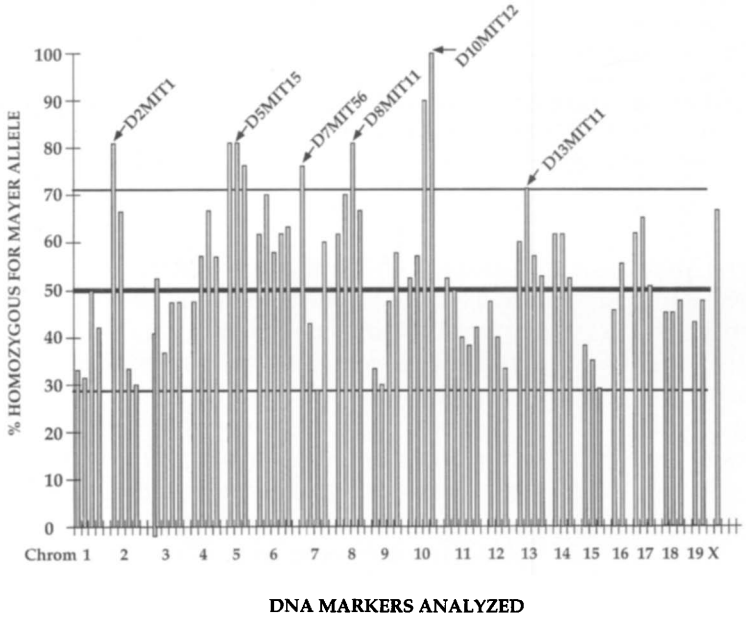

Figure 4 First level screen for modifier loci. The 21 whitest of the first 150 BC_1 animals were genotyped with the microsatellite markers shown in Fig. 2. The percentage of these mice that were homozygous for the Mayer allele (*y*-axis) was determined for each marker grouped by chromosome (*x*-axis). The six markers identified were homozygous for the Mayer allele in >72% of these progeny.

tical to that in the C3H *s/s* strain, the breeding records were not complete, and it was possible at the outset that the difference in severity of spotting was caused by different mutations at *s*. However, three lines of evidence argued against this possibility. First, the degree of spotting in the BC_1 animals showed a normal distribution with a mean value between the two parental means. If the phenotypic variation between the parental strains was entirely the consequence of two different *s* alleles, a bimodal curve with the peaks located over the parental means would have been observed. Second, Southern blot hybridization using EDNRB as a probe did not detect any restriction fragment length polymorphisms between the two strains (H. Rhim and W.J. Pavan, unpubl.). Third, no markers on mouse chromosome 14, where *s* maps, were linked to increased white spotting. Instead, strong linkage was established between increased spotting and loci on chromosomes 2, 5, 8, and 10; weaker linkage was also observed on chromosomes 7 and 13. Taken together, our findings demonstrate that the Mayer strain of *s/s* mice exhibit a more severe spotting pattern caused by the presence of multiple recessive modifiers unlinked to *piebald*.

The identification of the modifiers was accomplished by restricting the initial genotype analysis of the BC_1 animals to only the whitest animals. This approach has been exploited successfully in quantitative trait analysis in plants (Paterson et al. 1988; Lander and Botstein 1989). A PCR-based screen of the 21 whitest backcross progeny with microsatellite markers spanning the mouse genome in a 20-cM sweep radius identified six loci that demonstrated potential linkage to increased spotting. By limiting the genotype determination to only 21 of the 291 BC_1 animals, the

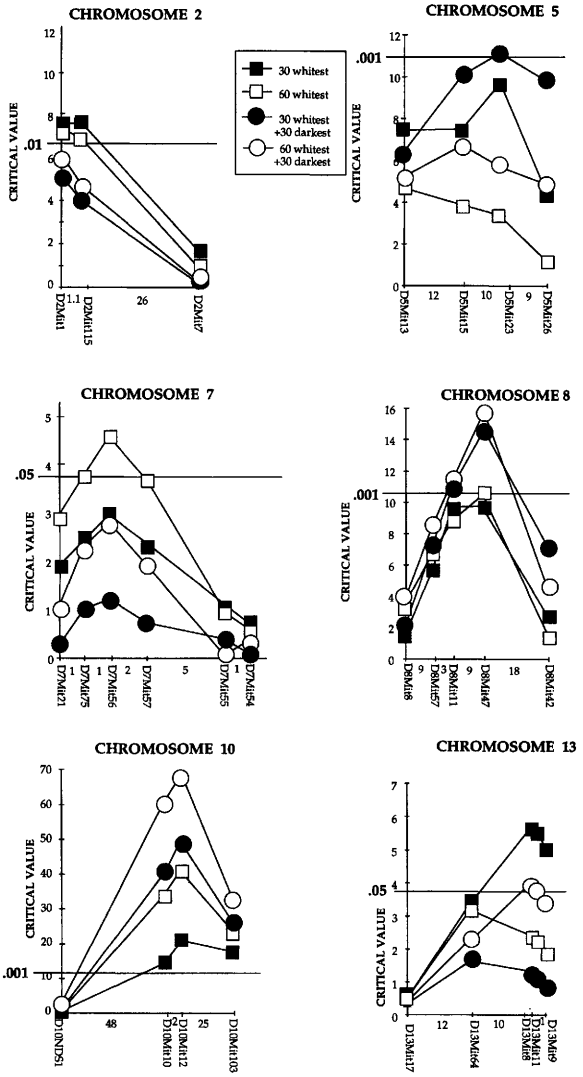

Figure 5 Second-level analysis of the six candidate modifier loci. Linkage was assessed by typing the 60 whitest and 30 darkest backcross progeny with the markers indicated. The critical values from the X^2 analyses and corresponding *P* values (*y*-axis) are plotted against the microsatellite markers (*x*-axis). The relative order and recombination distances between markers (indicated between markers in cM) were determined from these 90 animals.

amount of PCR typing was reduced by 10-fold. The limitation of this approach is that fluctuations that can occur at the extremes of the distribution may have resulted in the identification of false-positive modifier loci, and regions containing modifier loci may have been rejected. However, four of the loci (chromosomes 2, 5, 8, and 10) identified in the primary screen maintained their strong linkage when the entire BC_1 panel was tested; the remaining two loci (chromosomes 7 and 13) demonstrated strong linkage upon examining the likelihood of linkage using recombinant animals (Fig. 6). The 26 BC_1 animals that were homozygous for the Mayer allele for loci on chromosomes 2, 5, 8, and 10 exhibited a mean value for spotting (49 ± 10%) that was very similar to the parental Mayer strain (53 ± 7%). That is, these four loci account for 92.4% of the increase in spotting observed in the Mayer strain relative to the C3H strain. Furthermore, the eight BC_1 animals homozygous for the Mayer allele at all six identified loci exhibited a mean value for spotting of 53.9 ± 10%, indistinguishable from the parental Mayer strain.

The strongest modifier identified in the backcross was linked to D10Mit12 on chromosome 10. A highly plausible candidate gene for this modifier is *Mgf*, encoding mast cell growth factor (Anderson et al. 1990; Copeland et al. 1990; Huang et al. 1990; Nocka et al. 1990; Williams et al. 1990; Zsebo et al. 1990; Flanagan et al. 1991), which has been placed on the composite mouse genetic map distal to D10Mit12 (Copeland et al. 1993; Taylor et al. 1993). This growth factor, which is also referred to as *Steel* (*Sl*) factor because of the identity between *Mgf* and the *Sl* locus, is the ligand for the tyrosine kinase receptor, c-*kit*, the product of the *W* gene (Chabot et al. 1988; Geissler et al. 1988). Mutations in *Sl* have pleiotropic effects that include anemia, sterility, and disruption of melanocyte development, resulting in spotting (Bennett 1956). The *Sl* gene product is thought to be involved in the survival and secondary migration of melanoblasts (Steel et al. 1992). We have shown recently that *piebald* acts earlier than Steel, before embryonic day 10.5 (e10.5), by affecting the number of melanoblast precursor cells (Pavan and Tilghman 1994).

Should *Mgf* prove to be the *piebald* modifier on chromosome 10, it would resolve a long-standing mystery regarding the cell-autonomous action of *piebald*. Mayer used his highly spotted strain of *s/s* mice in transplantation experiments in which wild-type and *s/s* embryonic neural crest were grown in the presence of embryonic skin from the same sources (Mayer 1967a,b). He found that the neural crest from *s/s* mice was deficient in supporting the development of melanocytes, even in the presence of wild-type skin, suggesting a defect in the neural crest cells. However, skin from e11.0 to e15.5 *s/s* embryos appeared to contain an inhibitor that affected melanocyte development from *s/s* neural crest but not wild-type neural crest. He suggested that the

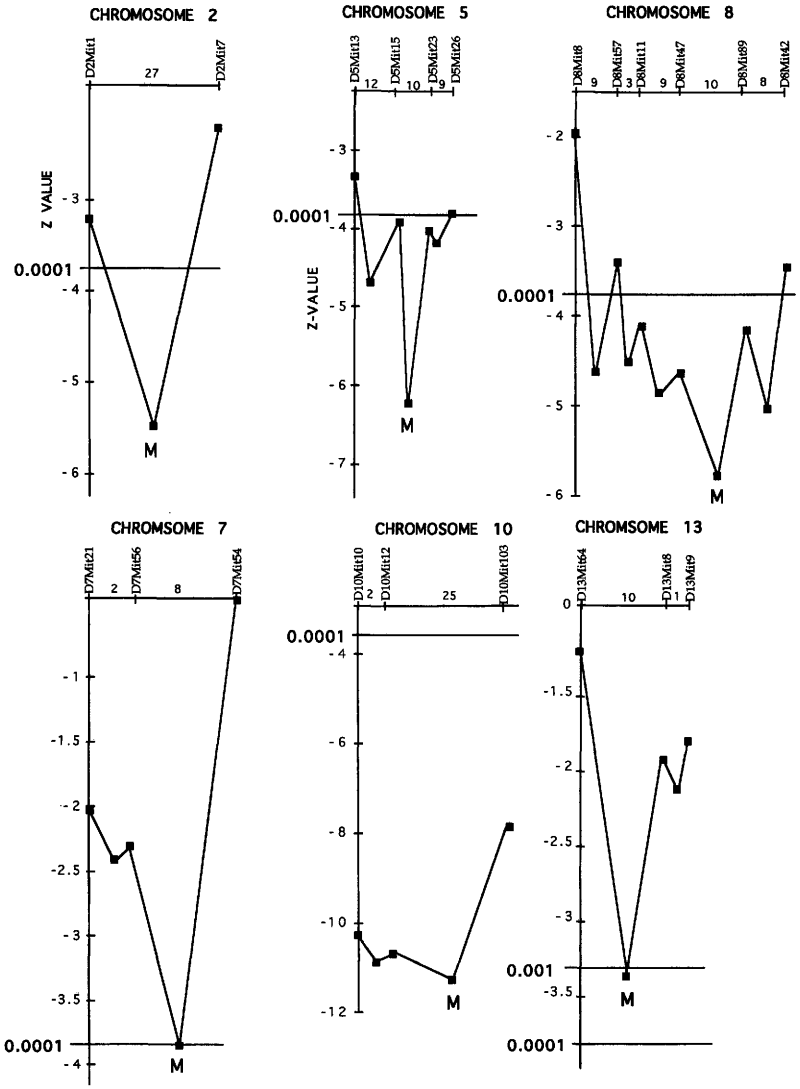

Figure 6 Chromosome position of modifier loci. The 125 whitest and 125 darkest BC_1 progeny were genotyped using linked markers near each candidate locus. The probability that the modifier loci (M) are positioned at or between each marker was determined using Mann–Whitney U test. The Z values of the Mann–Whitney U test (*y*-axis) are plotted against the microsatellite markers typed (*x*-axis). The recombination distances are indicated between markers in cM.

s gene product acted both in the neural crest and the skin, or that modifier loci were acting in the skin. Our study supports the latter conclusion. The fact that *Sl* has been shown not to have a cell-autonomous action in the skin between e12.5 and e15 increases the likelihood that it is responsible for the modifier effect on chromosome 10 (Mayer and Green 1968; Mayer 1970).

The modifier on chromosome 10 was the only one that showed a spatially restricted mode of action, with a greater effect on dorsal spotting than on ventral spotting. The neural crest precursors to melanocytes arise at the dorsal edges of the neural folds and migrate in a dorsal-to-ventral pattern during embryogenesis (Rawles 1947). One explanation for the restricted action of this modifier is that the expression of the gene product may be required only on the dorsal surface. This scenario would be difficult to reconcile with the phenotype of weak *Sl* alleles, which affect the ventral surfaces more severely than the dorsal surfaces (Silvers 1979). Alternatively, the gene product may be required throughout the skin, but the allele in Mayer's strain may affect the timing of expression or spatial distribution, such that its expression is delayed or decreased on the dorsal surface. Such an explanation could accommodate more readily *Sl* as the modifier.

The next strongest modifier segregates with D8Mit47 on chromosome 8. The composite map of that chromosome places D8Mit47 near the *e*, or extension, locus, which encodes the melanocyte-stimulating hormone (MSH) receptor (Robbins et al. 1993). Null mutations at *e* result in a decrease in the ratio of eumelanin to phaeomelanin in melanocytes, leading to a reddening or yellowing of the coat color. Consistent with *e* as a modifier, interactions between *s* and *e* loci have been suggested by the observation that *piebald* mice containing mutations in *e* had a reduced amount of spotting in comparison with those with a wild-type allele (Lamoreux and Russell 1979). Like the *piebald* gene product, the MSH receptor is a member of the G-protein-coupled, seven-transmembrane receptor family (Robbins et al. 1993). Should the *e* locus be confirmed as a modifier of *piebald* spotting, it would suggest an interaction among these gene products.

The third modifier for which candidate genes are suggested by map position is located between D5Mit15 and D5Mit23 on chromosome 5. D5Mit15 maps within 1 cM of a cluster of three dominant spotting mutations, *W* (c-*kit*), *Patch*

Table 3. Statistical analysis of candidate modifier loci

	Ventral[a]	Dorsal[a]	Total[a]	Contribution to total spotting[b] (%)
D2MIT1	0.001	0.004	0.001	12.4
D5MIT23	0.0001	0.001	0.0001	15.5
D7MIT56	0.02	0.03	0.02	9.1
D8MIT47	<0.0001	0.0003	<0.0001	18.2
D10MIT12	<0.0001	<0.0001	<0.0001	39.8
D13MIT8	0.02	0.18	0.06	7.2

[a]The Mann-Whitney U test was used to calculate the likelihood of linkage of the markers indicated to an increase in dorsal, ventral, and total spotting, based on the analysis of the 291 backcrossed animals.
[b]The contribution to total spotting was calculated as the difference between the means of animals homozygous and heterozygous for that locus, divided by the difference between the means of the Mayer and F_1 *s/s* strains × 100.

(platelet-derived growth factor receptor), and *Rump white*, whose gene defect has not been identified (Kozak and Stephenson 1993; Nagle et al. 1994; Stephenson et al. 1994). In addition, *Mgsa*, encoding melanoma growth stimulatory activity, maps between the two markers (Seldin et al. 1990). Melanoma growth stimulatory activity, also referred to as GRO protein or macrophage inflammatory protein, is a member of the chemokine superfamily of growth and inflammation regulators. Although the levels of MGSA/GRO are low is adult melanocytes, they are elevated in melanoma cells (Bordoni et al. 1990; Rodeck et al. 1991). Its expression during embryogenesis has not been reported.

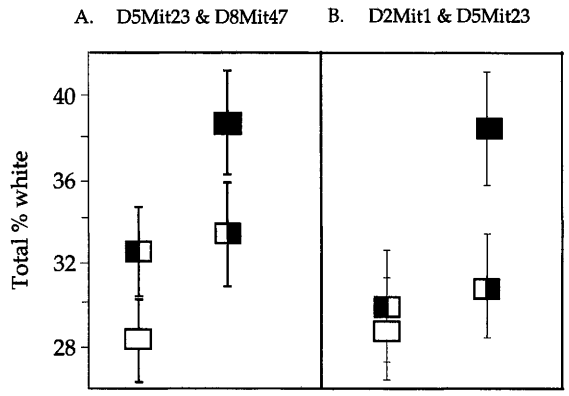

Figure 7 Genetic interactions among modifier loci. (*A*) The mean percent of white spotting of 77 animals who were heterozygous for both D5Mit23 and D8Mit47 (open square), the 69 animals homozygous for D5MiT23 and heterozygous for D8Mit47 (*left* half-filled square), the 71 animals heterozygous for D5Mit23 and homozygous for D8Mit47 (*right* half-filled square), and the 74 animals homozygous for both loci (solid square) are indicated. (*B*) The average percent of white spotting of 75 animals who were heterozygous for both D2Mit1 and D5Mit23 (open square), the 68 animals homozygous for D2Mit1 and heterozygous for D5Mit23 (*left* half-filled square), the 72 animals heterozygous for D2Mit1 and homozygous for D5Mit23 (*right* half-filled square), and the 76 animals homozygous for both loci (solid square) are indicated. The error bars represent the 95% confidence interval.

Nonadditive interactions were observed only between the modifiers on chromosomes 5 and 2, for which no candidate genes are suggested. Mice that were heterozygous for one of those modifiers did not show any increase in spotting over animals that were heterozygous for both loci. Only animals that were homozygous for both modifiers demonstrated a significant increase in spotting. This observation may explain the lower values for linkage observed with these loci. The finding of nonadditive interactions between loci suggest that they act together to affect melanocyte development. This could result from a direct interaction between the two gene products or redundant functions in the same pathway.

Mice carrying the hypomorphic *s* allele rarely develop aganglionic megacolon because of an abnormal development of the neural crest-derived enteric ganglia in the distal bowel. However, *piebald lethal* mice, which are null for the gene, are fully penetrant for this trait (Lane 1966). A congenital disorder in humans, Hirschsprung disease, is characterized by aganglionic megacolon and hypopigmentation, and the human EDNRB gene has been implicated in a subset of families with the disorder (Puffenberger et al. 1994). Although the highly spotted Mayer strain does not exhibit an increased incidence of megacolon, further analyses need to be performed to determine whether any of the modifier loci alter

Table 4. Spatial effects of modifier loci

	Increase (%)[a]		*Dorsal ≠ ventral*	
	ventral	dorsal	P^b	P^c
D2MIT1	4.10	5.00	0.6811	0.6254
D5MIT23	6.30	5.00	0.0801	0.0962
D7MIT56	3.20	3.20	0.0777	0.4555
D8MIT47	7.00	6.30	0.245	0.1655
D10MIT12	9.10	19.60	<0.0001	<0.0001
D13MIT8	3.40	2.00	0.0602	0.1362

[a]The increase in either dorsal or ventral spotting was calculated as the difference between the mean spotting of animals homozygous and heterozygous for that locus.
[b,c]The statistical significance of the differences between dorsal and ventral spotting attributable to a single locus was calculated using either the Paired Sign test[b] or the Wilcoxon Signed Rank test[c]. For either method, the values for dorsal and ventral spotting for each mouse were represented as the difference from the mean of animals heterozygous for that locus.

the development of the neural crest-derived enteric nervous system.

In summary, this study illustrates the power of QTL analysis to identify genes that contribute to the severity of spotting in *piebald* mice. It is conceptually analogous to suppressor and enhancer screens in more genetically tractable organisms such as *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and *Caenorhabditis elegans*. One limitation of QTL, however, is that the number of modifiers that can be identified depends on the number of allelic differences among the strains. That is to say, genes that are identical in the two strains will not be revealed by this cross. For example, one might have anticipated that the *lethal spotting* (*ls*) gene might have been identified, as it codes for endothelin-3, whose disruption leads to a phenotype identical to that of *piebald* (Greenstein-Baynash et al. 1994). Nevertheless, localization of new and existing genes that contribute to melanocyte development through interactions with *piebald* is an important step in fully defining the melanocyte genetic pathway.

METHODS

Strains of *s/s* Mice

Two strains of *s/s* mice were used for these studies (Fig. 1). The strain C3HeBFeJ Le *a/a s/s* (C3H *s/s*) exhibits a low percent of spotting on its dorsal and ventral surfaces and was provided by Dr. Nancy Jenkins (Frederick National Cancer Institute, Frederick, MD). The C3H *s/s* mice were generated by crossing *s* onto C3HeBFeJ/Le *a/a* for 16 backcross generations and has been maintained subsequently by intercrossing. A second strain of *s/s* mice that exhibits a high degree of spotting was kindly provided by Dr. Thomas Mayer at Rider College (Mayer 1965). The strain was generated by selecting highly spotted *s/s* mice on an unknown genetic background. These mice have been bred selectively for a high percentage of white spotting for >20 years.

Phenotypic Analyses

Backcross and control animals were killed by CO_2 asphyxiation at 5–8 weeks of age. Then, photographs were taken of their dorsal and ventral surfaces, and tail clippings and liver samples were removed for DNA analyses. Photographs were enlarged 200% using a photocopy machine; a planimeter was used to determine the total and spotted areas on the surfaces of each mouse. To control for variations in size among animals, the percent of white (the white area/the total area × 100) was calculated for the dorsal and ventral surfaces of each animal. Total spotting was calculated as the average of spotting of the dorsal and ventral surfaces.

Descriptive statistics and analyses of variance calculations were generated using the STATVIEW statistical analysis program (Abacus Concepts, Inc.). For graphic representations of the data in Figure 2, mice were placed into 5% incremental categories by percent of white. However for all calculations and genotype determinations, exact values were used. An estimate of the minimum number of modifiers involved in the severity of spotting in this cross was determined using the formula of Wright (1952):

$$n = (m_1 - m_2)^2/4(|V_{BC1} - V_{F1}|)$$

where n is an approximation of the minimum number of loci; m_1 and m_2 are the mean percent of white of the backcross progeny and F_1 hybrids respectively; $V_{BC1} - V_{F1}$ are the variances of the backcross progeny and F_1 hybrids, respectively. This value is most likely an underestimate of the number of loci modifying this trait in that this equation makes several assumptions, including the assumption that each modifier locus contributes equal and additive effects.

Genotypic Analysis

Genomic DNA for PCR analyses were prepared from mouse tail clippings. A 1-cm piece of tail was incubated in 800 μl of buffer made of 10 mM Tris-HCl (pH 8), 100 mM NaCl, 100 mM Na_2EDTA (pH 8), 0.5% SDS, and 0.2 mg/ml of

proteinase K at 55°C for 48 hr with occasional vortexing. Proteins were extracted with 400 µl of buffered phenol/chloroform (3:1) and centrifuged at 10,000*g* for 5 min. A 600-µl aliquot of the aqueous supernatant was transferred to a microcentrifuge tube containing 1 ml of 95% ethanol. The tubes were inverted several times and centrifuged immediately at 10,000*g* for 4 min to collect the precipitated DNA. The supernatant was decanted, and the DNA pellets were dried and resuspended in 100 µl of buffer made of 10 mM Tris-HCl (pH 8.0) and 1 mM Na_2EDTA.

Aliquots of DNA were diluted 1:150 with H_2O, and 5 µl (~5–50 ng) was used in a 20-µl reaction containing 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 6.6 mM each of a pair of primers, 2.5 mM each of the four deoxynucleotides, 0.5 units of AmpliTaq (Perkin-Elmer Cetus). Reactions were incubated in a Perkin-Elmer 9600 as indicated in Table 2. The PCR products were resolved by PAGE using 10% acrylamide, nondenaturing gels, and the DNA products were stained using ethidium bromide. The genotype of each animal was scored by comparison with PCR control reactions using DNA from C3H *s/s*, Mayer *s/s*, and F_1 *s/s* animals (see Table 1).

ACKNOWLEDGMENTS

We thank Dr. Thomas Mayer for insightful discussions on melanocyte development in *piebald* mice and for providing his strain of *s/s* mice, and Dr. Nancy Jenkins for the C3H *s/s* mice. We also thank Dr. Lee Silver for his assistance in the design of the mouse crosses and their analysis; Drs. Debra Nagle and Maja Bucan for providing mapping information of genes on mouse chromosome 5; and Drs. Tim O'Brien, Kristen Ardley, Janice Rossi, Soumitra Ghosh, David Koos, and members of S.M.T.'s laboratory for helpful discussions. S.M.T. is an investigator in the Howard Hughes Medical Institutes, and W.J.P. was supported by an American Cancer Society postdoctoral fellowship.

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Received May 9, 1995; accepted May 25, 1995.