The Murine Dilute Suppressor Gene *dsu* Suppresses the Coat-Color Phenotype of Three Pigment Mutations That Alter Melanocyte Morphology, *d*, *ash* and *ln*

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ABSTRACT

The murine dilute suppressor gene, *dsu*, was identified because of its ability to suppress the dilute coat color of mice homozygous for the retrovirally induced allele (d^v) of the dilute locus (*d*). *dsu* is unlinked to the *d* locus and has recently been shown to be semidominantly inherited. The dilute phenotype of *d/d* mice is the consequence of abnormal melanocyte morphology. While wild-type melanocytes are dendritic, *d/d* melanocytes are adendritic. *dsu* apparently suppresses the dilute phenotype by restoring normal melanocyte morphology. In addition to *d*, two other loci, ashen (*ash*) and leaden (*ln*), have been identified that produce a diluted coat color associated with adendritic melanocytes. Interestingly, *d* and *ash* are closely linked on chromosome *9* while *dsu* and *ln* are located on chromosome *1*. In experiments described here, we present genetic mapping data between *ash* and *d* indicating that, despite their identical phenotypes, they are separate genes and are not intragenic complementing alleles of the same locus. We also show that *dsu* is only loosely linked to *ln* (approximately 9 cM proximal) and that *dsu* can suppress, at least partially, the coat color of *ln/ln* mice and *ash/ash* mice. The partial suppression of *ln* and *ash* coat colors is associated with the partial restoration of normal melanocyte morphology. These studies provide new insights into the mechanism of action of *dsu* and into the interrelationships between members of a family of pigment genes.

THERE are more than 50 loci in the mouse that affect the pigmentation of the coat. This number is not surprising considering the many and varied developmental processes that culminate in visible pigmentation. These processes include the development of melanoblasts and their subsequent migration from the neural crest, the maturation of melanoblasts to melanocytes, pigment granule formation, and the subsequent movement of the granules out of the melanocyte and into the hair shaft.

Three of these known coat color loci, dilute (*d*), leaden (*ln*) and ashen (*ash*), have been grouped together because of their similar phenotypes. Mutations at these loci produce a diluting effect on hair pigmentation, and in each case, the dilute phenotype appears to be a consequence of abnormal melanocyte morphology (MARKERT and SILVERS 1956, LANE and WOMACK 1979). In contrast to wild-type melanocytes, which are highly dendritic, melanocytes of these mutant mice are virtually adendritic. Pigment granules produced in melanocytes are normally transported to the hair shaft via the dendrites. The loss of dendrites results in an inefficient transport of pigment granules into the hair shaft and causes an uneven distribution of the pigment granules and a lightening of the coat. There is no detectable change in the quantity of pigment synthesized by *d* mutant mice (RUSSELL, 1948) and no reports of such an alteration for either *ln* or *ash*.

dsu was first recognized by its ability to suppress the dilute coat color phenotype of mice homozygous for the retrovirally induced dilute (d^v) allele (SWEET 1983). Suppression is associated with the restoration of normal melanocyte morphology. However, suppression is not complete and on a nonagouti (*a/a*) background, *dsu/dsu*, d^v/d^v mice display an off-black phenotype that is distinguishable from a true nonagouti black mouse carrying wild-type alleles at *dsu* and *d* (K. J. MOORE, P. K. SEPERACK, M. C. STROBEL, D. A. SWING, N. G. COPELAND and N. A. JENKINS, unpublished results). While *dsu* was originally reported as being recessive, we have recently obtained evidence indicating that *dsu* acts semidominantly; different gene dosages of *dsu* can be identified by their differential effects on *d* (K. J. MOORE *et al.*, unpublished results). We have also shown that *dsu* can suppress the dilute coat color of a *d* allele, d^{120J}, which is caused by a 3.5-kb deletion (K. J. MOORE *et al.*, unpublished results). These studies suggest that *dsu* may either produce a mutant protein that can substitute for the

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absence of the *d* gene product(s) in melanocytes or, alternatively, that suppression results from the abnormal temporal or developmental expression of an otherwise normal *dsu* product.

In studies described here, we present the results of several genetic crosses designed to determine: (1) whether *d* and *ash* represent mutations at independent loci; (2) the map position of *dsu* on chromosome *1*, and (3) whether *dsu* can suppress the dilute coat color phenotype produced by *ln* and *ash*.

MATERIALS AND METHODS

Mice: All mice described in this study are maintained by the Mammalian Genetics Laboratory at the NCI-Frederick Cancer Research Facility.

Southern blot analysis: Genomic DNA was prepared from mouse tails (SIRACUSA *et al.* 1987). The DNA was digested with 10 units *Eco*RI/μg DNA (New England Biolabs) in a high salt buffer (MANIATIS, FRITSCH and SAMBROOK 1982) containing 5 mM spermidine. The digested DNAs were electrophoresed through 0.8% agarose gels and processed as described (JENKINS *et al.* 1982), except that Zetabind (AMF Cuno) was substituted for nitrocellulose. Hybridization conditions were as described (JENKINS *et al.* 1982). The DNA probe, pPS1.25, used for the detection of *Emv-17*, is a 1.2-kb *Pst*I-*Sst*I unique sequence fragment derived from cellular sequences located 5′ to the *Emv-17* viral integration site (BUCHBERG *et al.* 1986). This probe detects an 18.4-kb *Eco*RI fragment in DNA of RF/J mice and mice derived from this strain that carry the *Emv-17* provirus. It detects a 9.6-kb *Eco*RI fragment in DNA of C57L/J mice and mice of the *dsu* stock, which both lack the *Emv-17* provirus. For the differentiation of d^v and + alleles of *d* we used the probe p0.3 (originally published as the Pst probe; COPELAND, HUTCHINSON and JENKINS 1983), a unique 2.6-kb cellular DNA sequence located 3′ of the *Emv-3* viral integration site in the d^v allele (RINCHIK *et al.* 1986). This probe detects an 18-kb *Eco*RI fragment in d^v chromosomes and a 9-kb fragment in wild-type chromosomes. Filters were washed twice at 65°C in a shaking water bath with 2× SSCP, 0.1% SDS for 20 min each, then washed 3–4 times with 0.2× SSCP, 0.1% SDS for 20 min each. Filters were autoradiographed at −70°C with Kodak XAR film with 2 Dupont Lightning Plus intensifying screens for 1–5 days.

***Idh-1* typing:** A small lobe of liver was removed from the euthanized mouse and homogenized in 5× the volume/weight in ice cold 50 mM Tris buffer pH 8.0, 1 mM EDTA, 0.1% Triton. One ml of the homogenate was spun in an Eppendorf microfuge for 15 min at 4°C. The cleared supernatant fluid was applied directly on Titon III cellulose acetate plates (Helena Laboratories) and run for 30 min at 200 V in a Helena Zip Zone Apparatus. The electrophoresis buffer used was a Tris/citrate buffer at a final concentration of 20 mM Tris, 9 mM citrate, pH 6.2. The enzyme-specific stain for *Idh-1* was as described (HARRIS and HOPKINSON 1976) but was prepared in 0.75% agar. The agar/stain mix was poured over the Titan III plates and incubated in the dark, at 37°C, until the bands of enzyme activity developed.

Preparation of Harderian glands: Ten- to 12-day-old mice were euthanized and the Harderian glands, still attached to the eye, were removed. The dissected tissues were fixed in Fekete Tellyesniczky's fluid (LILLY 1965) and prepared as described (SWEET 1983).

TABLE 1

Segregation of d^v and *ash* in reciprocal crosses of (C57BL/6J-d^v-*se*/d^v *se* × C3H/HeSn-*ash*/*ash*) × C3H/HeSn-*ash*/*ash* mice[a]

	Genotype	Phenotype	No. of Mice
Parental classes	$\frac{ash\ +}{ash\ +}$	Ashen	249
	$\frac{ash\ +}{+\ d^v}$	Wild type	227
Recombinant classes	$\frac{ash\ +}{ash\ d^v}$	Ashen	1
	$\frac{ash\ +}{+\ +}$	Wild type	4

Map distance between *ash* and *d* = 1.04 ± 0.90 cM

[a] C3H/HeSn-*ash*/*ash* mice are genotypically *ash* + +/*ash* + + whereas C57BL6/J-d^v *se*/d^v *se* mice are + d^v *se*/+ d^v *se*.

RESULTS

Linkage analysis of *d* and *ash*: The original chromosome mapping of *ash* was done using the linked electrophoretic markers *Lap-1*, *Sep-1* and *Mod-1* (LANE and WOMACK 1979). This study placed the *ash* gene very close to *d* on chromosome *9*. Allelism testing of ashen mice to dilute mice showed that these two mutations complemented each other, suggesting that *d* and *ash* are separate genes. However, as both mutants produce indistinguishable phenotypes, it is possible that the two mutations define intragenic complementing regions of a single gene rather than two separate genes. To examine this question further, we have directly measured the genetic distance between *ash* and *d*.

C3H/HeSn-*ash*/*ash* and C57BL/6J-d^v *se*/d^v *se*[a strain homozygous for d^v and a very closely linked gene short-ear (*se*), which is less than 0.2 cM from *d* (RUSSELL 1971)] mice were crossed. The resulting F_1 mice (+ d^v *se*/*ash* + +) were backcrossed to C3H/HeSn-*ash*/*ash* mice. The backcross progeny fell into two phenotypic classes: (1) those that were wild-type in color and (2) those that were ashen in color. All progeny were analyzed for the presence of d^v by Southern blot analysis with the p0.3 probe. The results of this cross are summarized in Table 1. The *se* mutation was not typed in this cross although most, if not all, progeny carrying d^v should also carry *se* due to the close linkage of these genes. Of 481 mice analyzed, 5 carried recombinant chromosomes. Four of these recombinant chromosomes carried wild-type alleles at *d* and *ash*. The fifth recombinant carried both *ash* and d^v on the same chromosome. These data give a recombination distance of 1.04 ± 0.90 cM (95% confidence interval) between *ash* and *d*, strongly suggesting that they are two distinct genes.

The one recombinant mouse that carried *ash* and d^v (and also *se*, data not shown) on the same chromo-

TABLE 2

Comparative coat color of mice carrying various combinations of pigment genes with and without the *dsu* suppressor

Genotype[a]	Phenotype
ash d^v/*ash* d^v	Light gray
ash/*ash* d^v/d^v *ln*/*ln* *ln*/*ln*, d^v/d^v	Gray
dsu/+, d^v/d^v	Dark gray
dsu/*dsu*, *ash*/*ash* *dsu*/*dsu*, *ash* d^v/*ash* d^v *dsu*/*dsu*, *ash* d^v/*ash* + *dsu ln*/*dsu ln*, d^v/d^v *dsu ln*/*dsu ln*	Light off-black
dsu/*dsu*, d^v/d^v	Off-black
dsu/*dsu*, d^v/+ *dsu*/*dsu*, +/+	Black

[a] With the possible exception of d^v/d^v, *ln*/*ln* mice, all of the genotypes were constructed on a nonagouti background. The coat color of d^v/d^v, *ln*/*ln* mice was previously reported (SILVERS 1979) and was not determined in these experiments. In some cases, these mice also carried *se* or *Emv-17* alleles as described in the text.

TABLE 3

Suppression of the *ash* d^v coat color phenotype by *dsu*

$$\frac{+}{+}\ \frac{ash\ d^v\ se}{ash\ d^v\ se} \times \frac{dsu}{dsu}\ \frac{+\ d^v\ +}{+\ d^v\ +}$$

$$\downarrow$$

$$\frac{+}{dsu}\ \frac{ash\ d^v\ se}{+\ d^v\ +}$$

1. Intercross and select short-eared progeny

Phenotype	Presumed genotype[a]	No. of short-eared mice[b]	No. mice expected[c]
Light gray, short-eared	$\frac{+}{?}\ \frac{ash\ d^v\ se}{ash\ d^v\ se}$	20	24
Light off-black, short-eared	$\frac{dsu}{dsu}\ \frac{ash\ d^v\ se}{ash\ d^v\ se}$	9	8

[a] Presumed genotype in the absence of recombination between *ash*, d^v, and/or *se*. The ? indicates that the *dsu* genotype of one homolog was not determined.

[b] These figures are based on a total of 130 F_2 mice characterized.

[c] Number of mice expected assuming that *dsu* suppresses the *ash* d^v double mutant phenotype, as observed. The number of mice found did not differ significantly from the number of mice expected ($\chi^2_2 = 0.92$; $0.9 > P > 0.5$).

some was used as the founder to produce a stock homozygous for *ash*, d^v and *se*. This stock was also fixed for the nonagouti (*a*) allele at the agouti locus on chromosome 2 [C57BL/6J mice are nonagouti (*a*/*a*) while C3H/HeSn-*ash*/*ash* mice are agouti (*A*/*A*)]. All mice used in subsequent suppression studies (described below) were also fixed for nonagouti thereby allowing the visualization of all phenotypes on the same background. When visualized on the nonagouti background, the color of *ash* d^v *se*/*ash* d^v *se* mice was lighter than either *ash*/*ash* or d^v/d^v mice alone (Table 2). Thus, there appears to be a dosage effect of these two genes on coat color.

The interaction of *dsu* with *ash*: To determine whether *dsu* can suppress the coat color phenotype of ashen mice in a fashion similar to its suppression of *d*, we took advantage of the availability of the *ash* d^v *se*/*ash* d^v *se* stock. The presence of *dsu* cannot be followed through genetic crosses in the absence of *d* because *dsu* alone does not produce a distinct phenotype, *i.e.*, *dsu*/*dsu*, d^v/+ and *dsu*/*dsu*, +/+ mice are black (on a nonagouti background) (Table 2). Therefore, in the initial crosses, we introduced the *dsu* gene into the *ash* d^v *se* stock so we could follow *dsu* and, simultaneously determine whether *dsu* can suppress the *ash* d^v double coat color mutant. Subsequently, we bred out the d^v allele to examine directly the interaction of *dsu* and *ash*.

The first of these studies involved crossing mice homozygous for *ash* d^v *se* with *dsu*/*dsu*, d^v/d^v mice (Table 3). The F_1 progeny (+/*dsu*, *ash* d^v *se*/+ d^v +) were intercrossed. Short-eared F_2 progeny were then selected, that barring recombination, were homozygous for the *ash* d^v *se* chromosome. The short-eared mice produced in this cross could be classified into two phenotypic classes, a light gray class similar to the original *ash* d^v *se* stock and a much darker class. The darker mice looked very similar to the off-black phenotype of *dsu*/*dsu*, d^v/d^v mice but were a little lighter (Table 2). We have designated this color as light off-black. Southern blot analysis with the p0.3 probe confirmed that the light off-black, short-eared F_2 mice were homozygous for d^v. These mice were also shown to be homozygous for *ash* by test crossing to C3H/HeSn-*ash*/*ash* mice (data not shown). *dsu*/*dsu*, *ash* d^v *se*/*ash* d^v *se* mice were produced at the expected frequency (Table 3). A line of mice carrying the *dsu*/*dsu*, *ash* d^v *se*/*ash* d^v *se* genotype was subsequently established.

At the time of these experiments, we assumed that *dsu* was recessive, as originally reported (SWEET 1983). The subtle phenotypic distinction between the presence of zero and one copies of *dsu* noticed in subsequent experiments with *d* (K. J. MOORE *et al.*, unpublished results) had not been noticed prior to the experiments presented in Table 3. Therefore, mice from both genotypes, +/+, *ash* d^v*se*/*ash* d^v*se* and +/*dsu*, *ash* d^v*se*/*ash* d^v*se*, are listed as one class (light gray, short-eared) in Table 3. Subsequently, we noticed the subtle phenotypic difference between these two genotypic classes of progeny, consistent with the semidominant action of *dsu*.

Using the *dsu*/*dsu*, *ash* d^v *se*/*ash* d^v *se* mice, it was then possible to remove d^v to establish a *dsu*/*dsu*, *ash*/*ash* line. The breeding scheme used to produce these mice is described in Table 4. The *dsu*, *ash* d^v *se* mice

TABLE 4

Suppression of the *ash* coat color phenotype by *dsu*

$$\frac{dsu}{dsu}\,\frac{ash\ d^v\ se}{ash\ d^v\ se} \times \frac{+}{+}\,\frac{ash\ +\ +}{ash\ +\ +}$$

↓

$$\frac{dsu}{+}\,\frac{ash\ d^v\ se}{ash\ +\ +}$$

1. Backcross to light off-black, short-eared parent
2. Select for nonagouti[a], long-eared, light off-black progeny
3. Barring recombination between d^v and *se* and assuming that *ash* is suppressed by *dsu*, these mice should be the following genotype:

$$\frac{dsu}{dsu}\,\frac{ash\ d^v\ se}{ash\ +\ +}$$

1. Intercross

Phenotype	Presumed genotype[b]	No. mice found[c]	No. mice expected[d]
Light off-black, short-eared	$\frac{dsu}{dsu}\,\frac{ash\ d^v\ se}{ash\ d^v\ se}$	9	14
Light off-black, long-eared	$\frac{dsu}{dsu}\,\frac{ash\ d^v\ se}{ash\ +\ +}$	33	27
	$\frac{dsu}{dsu}\,\frac{ash\ +\ +}{ash\ +\ +}$	12	14

[a] The *dsu*, *ash* d^v *se* stock is nonagouti (*a/a*). C3H/HeSn-*ash/ash* mice are agouti (*A/A*).

[b] Presumed genotype in the absence of recombination between *ash*, d^v, and/or *se*.

[c] The presence of the d^v allele was determined by Southern blot analysis as described in MATERIALS AND METHODS.

[d] Number of mice expected assuming that *dsu* suppresses the *ash* coat color phenotype, as observed. The number of mice found did not differ significantly from the number of mice expected ($\chi^2_2 = 3.0$; $0.5 > P > 0.1$).

were crossed to C3H/HeSn-*ash/ash* mice. The F_1 progeny were then backcrossed to the *dsu*, *ash* d^v *se* parent. As the C3H/HeSn-*ash/ash* strain is agouti (*A/A*), 50% of the backcross progeny were *A/a* and phenotypically agouti. These mice were excluded from further analysis. Of the remaining 50% of the mice, which were nonagouti (*a/a*), half were short-eared (*ash* d^v *se/ash* d^v *se*, barring recombination). These mice were also excluded from further analysis. The remaining 25% of the mice (nonagouti, long eared and barring recombination, *ash* d^v *se/ash* + +) fell into two phenotypic classes: (1) light gray and presumably *dsu/+*; and (2) light off-black, which were presumably *dsu/dsu*. Mice of this latter class were intercrossed. The progeny should all be homozygous *ash* and *dsu* (barring recombination between *ash*, d^v, and/or *se*) and segregate for 0, 1, or 2 copies of d^v *se*. Those with two copies of *se* were identified by virtue of their short-eared phenotype. The other two classes were

TABLE 5

Derivation of recombinant chromosome *1* carrying *ln* and *Emv-17*[a]

$$\text{(RF/J)}\ \frac{Idh\text{-}1^a\ +\ Emv\text{-}17}{Idh\text{-}1^a\ +\ Emv\text{-}17} \times \text{(C57L/J)}\ \frac{Idh\text{-}1^b\ ln\ +}{Idh\text{-}1^b\ ln\ +}$$

↓

$$\frac{Idh\text{-}1^a\ +\ Emv\text{-}17}{Idh\text{-}1^b\ ln\ +}$$

1. Backcross to C57L/J parent
2. Select recombinant progeny of the following genotype

$$\frac{Idh\text{-}1^b\ ln\ Emv\text{-}17}{Idh\text{-}1^b\ ln\ +}$$

[a] The map location, gene order, and production of recombinant chromosome *1* was described previously (BUCHBERG *et al.* 1986).

typed by Southern blot analysis for their *d* locus genotype. Mice that were +/+ at *d* were selected and intercrossed to establish a breeding stock, of the *dsu/dsu*, *ash/ash* genotype. Phenotypically, this stock is light off-black and looks identical to the *dsu/dsu*, *ash* d^v *se/ash* d^v *se* stock (Table 2).

The interaction of *dsu* with *ln*: The third gene that is similar in phenotype to *ash* and *d* is leaden (*ln*), which maps to chromosome *1*. *dsu* also maps to chromosome *1* but the genetic distance between these genes was undetermined at the start of this analysis. Experiments to examine the interaction of *dsu* and *ln* therefore involved producing a recombinant chromosome that carried both *ln* and *dsu*. As mentioned previously, in order to follow *dsu* through the genetic crosses it was important to maintain a genotype upon which *dsu* could be scored. Once again, we used d^v to follow *dsu*.

To follow the segregation of *ln* throughout the crosses we took advantage of a recombinant chromosome produced in this laboratory (BUCHBERG *et al.* 1986) that carried the murine endogenous ecotropic proviral locus *Emv-17* (inherited from RF/J mice) on the same chromosome as *ln* (see Table 5 for the breeding scheme used to generate this recombinant chromosome). *Emv-17* is located 3.6 cM distal to *ln* (BUCHBERG *et al.* 1986). Using a unique sequence probe flanking *Emv-17* (pPS1.25), the segregation of *Emv-17* was followed by Southern blot analysis and, barring recombination between these two loci, we could also follow the segregation of *ln*. The mouse carrying the recombinant (*ln Emv-17*) chromosome *1* was also homozygous for the *Idh-1*^{b} (isocitrate dehydrogenase-1) allele, inherited from C57L/J mice (Table 5). *Idh-1* maps 14 cM proximal to *ln* and was used in subsequent experiments (described below) to map the chromosomal location of *dsu*.

To determine if *dsu* suppresses *ln*, the mouse carrying the *ln Emv-17* recombinant chromosome was

TABLE 6

Suppression of the *ln*, d^v coat color phenotype by *dsu*[a]

$$\frac{Idh\text{-}1^b + ln\ Emv\text{-}17\ +}{Idh\text{-}1^b + ln\ +\ +} \times \frac{Idh\text{-}1^a\ dsu + +}{Idh\text{-}1^a\ dsu + +}\ \frac{d^v}{d^v}$$

1. Select progeny carrying *Emv-17*

$$\frac{Idh\text{-}1^b\ +\ ln\ Emv\text{-}17\ +}{Idh\text{-}1^a\ dsu +\ +\ d^v}$$

1. Backcross to *dsu* parent

Phenotype	Genotype	No. mice found	No. mice expected[b]
Gray	$\frac{Idh\text{-}1^?\ +\ ln?\ Emv\text{-}17?}{Idh\text{-}1^a\ dsu +\ +}\ \frac{d^v}{d^v}$	66	78
Black	$\frac{Idh\text{-}1^?\ dsu?\ ln?\ Emv\text{-}17?}{Idh\text{-}1^a\ dsu +\ +}\ \frac{+}{d^v}$	163	156
Off-black	$\frac{Idh\text{-}1^?\ dsu\ ln?\ Emv\text{-}17?}{Idh\text{-}1^a\ dsu +\ +}\ \frac{d^v}{d^v}$	83	78

1. Type gray and off-black mice for *Emv-17* and *Idh-1* to map chromosomal location of *dsu* (see Table 8)
2. Select 12 off-black mice carrying *Emv-17* for further crosses (*Idh-1* genotype not important in subsequent crosses)

$$\frac{dsu\ ln?\ Emv\text{-}17}{dsu +\ +}\ \frac{d^v}{d^v}$$

1. Intercross and select homozygous *Emv-17* mice
2. Confirm homozygosity for *ln* by crossing to C57L/J mice

$$\frac{dsu\ ln\ Emv\text{-}17}{dsu\ ln\ Emv\text{-}17}\ \frac{d^v}{d^v}$$

[a] The gene order *Idh-1-dsu-ln-Emv-17* was determined as described (Table 8 and BUCHBERG *et al.* 1986). The ? indicates that the genotype was not determined. *Emv-17* and d^v were typed by Southern blot analysis. *Idh-1* was typed as described in MATERIALS AND METHODS.

[b] The number of mice found did not differ significantly from the number of mice expected ($\chi^2_2 = 2.5$; $0.5 > P > 0.1$).

crossed to *dsu/dsu*, d^v/d^v mice (Table 6). Mice heterozygous for *Emv-17* were then backcrossed to the *dsu/dsu*, d^v/d^v parent. Three phenotypic classes of progeny were obtained from this cross: (1) gray mice, which were *dsu/+*, d^v/d^v; (2) black mice, which were *dsu/+*, $d^v/+$ or *dsu/dsu*, $d^v/+$; and (3) off-black mice, which were *dsu/dsu*, d^v/d^v (Table 6). The off-black class was analyzed by Southern blot analysis using the *Emv-17* flanking probe to look for recombinant chromosomes that carried both *Emv-17* and *dsu*. Excluding recombination between *ln* and *Emv-17*, these mice would also carry *ln* (Table 6).

Mice that carried *dsu ln? Emv-17/dsu + +*, d^v/d^v (the ? indicates that the *ln* allele of these animals was undetermined) were intercrossed and the offspring were typed for *Emv-17*. Mice that were homozygous for *Emv-17* were tested to confirm that during the establishment of the *dsu Emv-17* recombinant chromosome, the *ln* allele was not lost. This was done by mating them to C57L/J mice, which are homozygous *ln*. If the *Emv-17* homozygotes have retained the *ln* allele on both recombinant chromosomes, all the offspring will be leaden in coat color; if only one of the recombinant chromosomes retained *ln* then half the offspring will be leaden and half will be black; and if both recombinant chromosomes lost the *ln* allele, all the offspring will be black. In order to calculate what proportion of chromosomes that carried *dsu* and *Emv-17* still carried *ln* the following facts were considered: (1) that the distance between *dsu* and *Emv-17* is 12.6 cM (Table 8, see below); (2) that *ln* is 3.6 cM proximal to *Emv-17* (BUCHBERG *et al.* 1986) placing *ln* between *dsu* and *Emv-17*; (3) that only the recombination events that took place proximal to the *ln* gene, within the *dsu-Emv-17* interval would result in a *dsu-ln-Emv-17* chromosome. The expected proportion of recombinant chromosomes that still carry *ln* is therefore (12.6 − 3.6)/12.6 or about 70%. Seven of the 12 (58%) recombinant chromosomes analyzed (Table 6) carried *ln*. Mice that were confirmed to be *ln/ln* and, in addition, were *dsu/dsu*, d^v/d^v, were light off-black in color and were similar in phenotype to *dsu/dsu*, *ash/ash* or *dsu/dsu*, *ash* d^v *se/ash* d^v *se* mice.

A protocol similar to that used for the removal of d^v from the *dsu*, *ash* d^v mice, was used to remove the d^v allele from the *dsu ln*, d^v mice. These crosses are described in Table 7. *dsu ln Emv-17*, d^v homozygotes were crossed to C57BL/6J mice. The F_1 progeny (*dsu ln Emv-17/+ + +*, $d^v/+$) were backcrossed to the *dsu ln Emv-17*, d^v parental mice. Mice showing the suppressed phenotype of light off-black (and therefore *dsu/dsu*) were selected and were analyzed for both the *d* and *Emv-17* genotypes. Mice that were *Emv-17* homozygotes and $d^v/+$ were selected and intercrossed. The offspring of this cross were analyzed for their *d* genotype and the +/+ mice were selected. These mice were *dsu Emv-17/dsu Emv-17*, +/+ (at *d*) and, barring the unlikely event of a double recombination between *Emv-17* and *dsu*, they would be homozygous *ln*. The *ln* genotype of these mice was confirmed by crossing to C57L/J mice. So far, two males have been analyzed and both have proved to be *ln/ln*. Both are a light off-black and are indistinguishable from mice homozygous for *dsu ln Emv-17*, d^v. Therefore, we conclude that *dsu* can partially suppress the leaden coat color phenotype in a manner similar to its suppression of *ash*.

Histological analysis of suppressed and nonsuppressed melanocytes: Dendritic melanocytes are

TABLE 7

Suppression of the *ln* coat color by *dsu*

$$\frac{dsu\ ln\ Emv\text{-}17}{dsu\ ln\ Emv\text{-}17}\frac{d^v}{d^v} \times (\text{C57BL/6J})\frac{+\ +\ +\ +}{+\ +\ +\ +}$$

$$\downarrow$$

$$\frac{dsu\ ln\ Emv\text{-}17}{+\ +\ +\ +}\frac{d^v}{+}$$

1. Backcross to the d^v, *dsu ln Emv-17* parent
2. Select the offspring for
   i) light off-black phenotype
   ii) *Emv-17/Emv-17*
   iii) $d^v/+$
3. Barring recombination these mice should be the following genotype

$$\frac{dsu\ ln\ Emv\text{-}17}{dsu\ ln\ Emv\text{-}17}\frac{d^v}{+}$$

1. Intercross

Phenotype	Genotype	No. mice found	No. mice expected
Light off-black	$\frac{dsu\ ln\ Emv\text{-}17}{dsu\ ln\ Emv\text{-}17}\frac{d^v}{d^v}$	4	5.5
Light off-black	$\frac{dsu\ ln\ Emv\text{-}17}{dsu\ ln\ Emv\text{-}17}\frac{d^v}{+}$	13	11.0
Light off-black	$\frac{dsu\ ln\ Emv\text{-}17}{dsu\ ln\ Emv\text{-}17}\frac{+}{+}$	5	5.5

1. Select for +/+ (at *d*)
2. Confirm the *ln/ln* genotype by test crossing to C57L/J mice

$$\frac{dsu\ ln\ Emv\text{-}17}{dsu\ ln\ Emv\text{-}17}\frac{+}{+}$$

found not only in hair follicles of mice but are also found in the dermis and epidermis of some areas of the body and in the connective tissue that encapsulates and subdivides the Harderian gland of the eye. Histological examination of Harderian gland melanocytes has been the method of choice for examining the aberrant melanocyte morphology of *d/d*, *ln/ln*, and *ash/ash* mice and for demonstrating that suppression of *d* by *dsu* is accompanied by the restoration of normal melanocyte morphology (SWEET 1983). To determine if the partial suppression of *ash* and *ln* by *dsu* is accompanied by a partial restoration of normal melanocyte morphology, we prepared Harderian gland sections from mice carrying several different pigment and *dsu* genotypes including: (a) wild type (C57BL/6J mice); (b) *dsu/dsu, ash* $d^v/ash\ d^v$; (c) d^v/d^v; (d) *dsu/dsu,* d^v/d^v; (e) *ash/ash*; (f) *dsu/dsu, ash/ash*; (g) *ln/ln* (C57L/J mice); and (h) *dsu ln/dsu ln,* d^v/d^v. Photomicrographs of these sections are shown in Figure 1. Wild-type melanocytes of C57BL/6J mice (panel a) have many long dendritic processes. Panels c, e and g show the melanocytes of d^v/d^v, *ash/ash*, and *ln/ln* mice, respectively. They all show a similar adendritic morphology. Panels d, f and h show melanocytes from mice of the same (or similar) genotypes with the addition of *dsu*. In each case the melanocytes have a dendritic morphology due to the suppression of *dsu*. The suppression is most pronounced in *dsu/dsu,* d^v/d^v mice (panel d) and is consistent with the observation that coat color suppression is most complete in *dsu/dsu,* d^v/d^v mice (Table 2). In contrast, suppression in *dsu/dsu, ash/ash* (panel f) and *dsu ln/dsu ln,* d^v/d^v (panel h) mice is less complete, a finding consistent with the observation that coat color phenotype is also less suppressed in these mice (Table 2). Partial suppression was also observed for melanocytes of *dsu/dsu, ash* $d^v/ash\ d^v$ mice (panel b). These data suggest that the level of coat color suppression by *dsu* is directly related to the ability of *dsu* to restore normal melanocyte morphology.

Mapping *dsu*: In a previously reported study (SWEET 1983) *dsu* was mapped to chromosome *1*, 6.3 ± 3.5 cM from *Idh-1*. However, its proximal or distal relationship to *Idh-1* was not established and it was not directly tested to determine whether it can recombine with *ln*. The breeding scheme described in Table 6, which generated recombinant chromosomes carrying *dsu, ln* and *Emv-17* gave data to map *dsu* in a three point cross. It also confirmed that *dsu* and *ln* are two separate loci, as originally thought. The three markers used in the mapping experiments were *Idh-1, dsu* and *Emv-17* (Table 6). *ln* was not used as a marker in these mapping studies, because the *ln* genotype of each backcross segregant could only be determined by progeny testing. Fifty percent of the backcross mice described in Table 6 are black and could be either *dsu/dsu,* $d^v/+$ or *dsu/+,* $d^v/+$. Since their *dsu* genotype was undetermined, these mice were not used in the mapping studies. The remaining 50% of the mice, which were either dark gray (*dsu/+,* d^v/d^v) or off-black (*dsu/dsu,* d^v/d^v), were typed for *Idh-1*, and the segregation data are summarized in Table 8. From this analysis we were able to obtain a gene order for these three loci of centromere-*Idh-1*-6.3 cM-*dsu*-12.6 cM-*Emv-17*. This gene order is 1.9×10^5 times more likely than the next most likely gene order centromere-*dsu-Idh-1-Emv-17* (BISHOP 1985). As *ln* is 3.6 cM proximal to *Emv-17* the distance between *dsu* and *ln* is about 9.0 cM.

DISCUSSION

Several genetic crosses are reported indicating that *dsu* can suppress the dilute coat color of *ln/ln* mice and *ash/ash* mice in addition to that of d^v/d^v mice. While all three mutations have identical coat color phenotypes, they are not suppressed to the same ex-

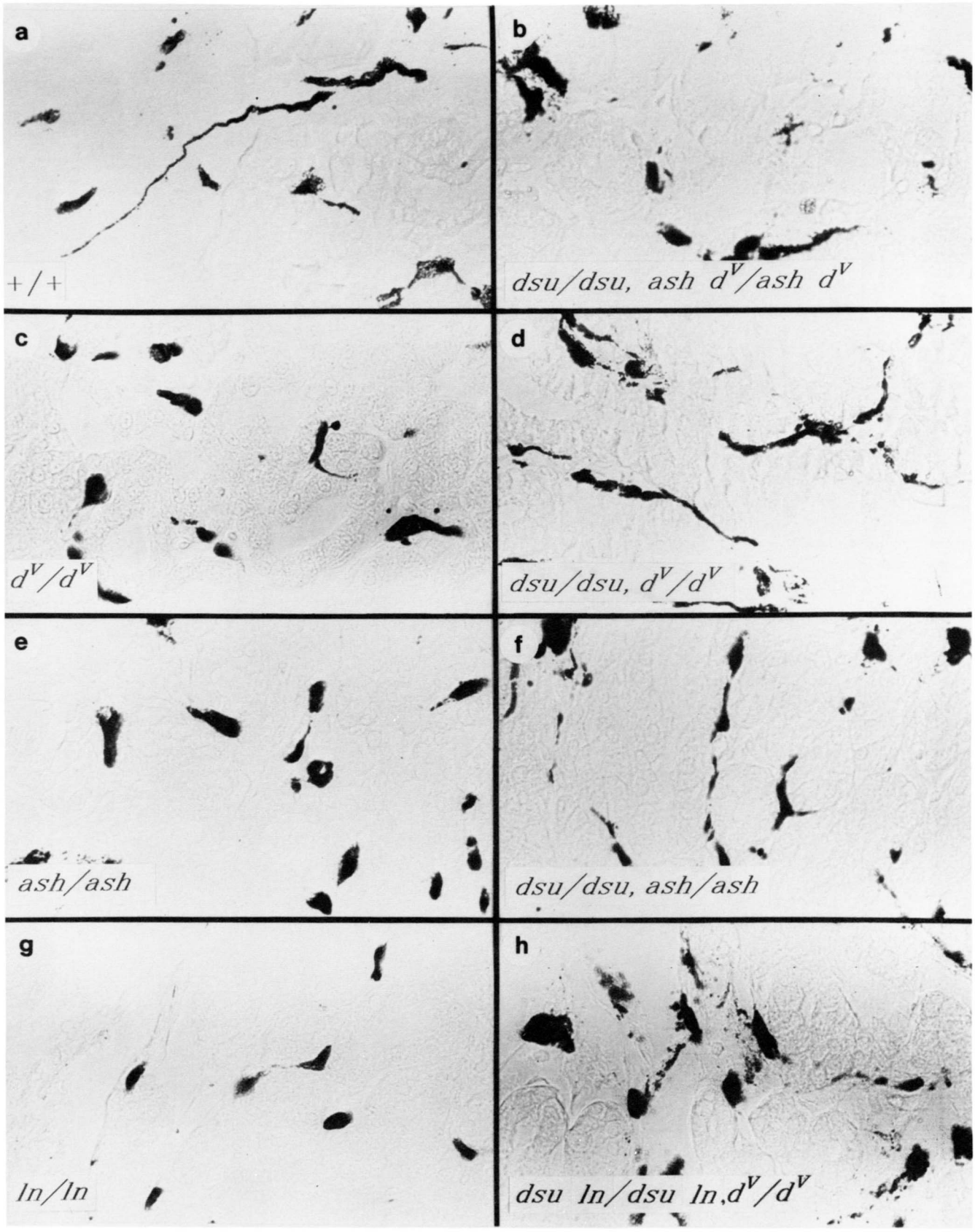

FIGURE 1.—Sections of unstained Harderian glands from 10- to 12-day-old mice carrying various combinations of pigment genes with and without the *dsu* suppressor gene. Only the appropriate pigment and suppressor gene symbols are shown; *dsu/dsu*, *ash* d^v/ash d^v mice were also homozygous for *se* and *dsu ln/dsu ln*, d^v/d^v mice were homozygous for *Emv-17*. In addition, all mice were homozygous for nonagouti (*a/a*).

TABLE 8

Segregation of parental and recombinant chromosomes in a three-point test cross to determine the chromosomal location of *dsu*[a]

Chromosome	Genotype	No. of mice
Parental	*Idh-1*[b] + *Emv-17*	53
	Idh-1[a] *dsu* +	64
Recombinant	*Idh-1*[a] *dsu Emv-17*	10
	Idh-1[b] + +	7
	Idh-1[a] + *Emv-17*	5
	Idh-1[b] *dsu* +	3
Double recombinant	*Idh-1*[b] *dsu Emv-17*	0
	Idh-1[a] + +	1
Map distance:	*dsu* − *Emv*-17 (12.6 ± 5.5 cM)	
	Idh-1 − *dsu* (6.3 ± 3.9 cM)	
	Idh-1 − *Emv*-17 (18.3 ± 6.3 cM)	

[a] See Table 6 for the breeding program used to generate the parental and recombinant chromosomes.

tent. Suppression of d^v by *dsu* is almost complete; the mice are nearly black and are referred to as off-black. On the other hand, suppression of *ash* and *ln* by *dsu* is less complete, and the mice are referred to as light off-black. Melanocytes from *dsu/dsu*, d^v/d^v mice are dendritic, and appear indistinguishable from wild-type melanocytes. Melanocytes of *dsu/dsu*, *ash/ash* (and *dsu/dsu*, *ash* d^v/*ash* d^v) or *dsu ln/dsu ln*, d^v/d^v (and presumably *dsu ln/dsu ln*) mice are not as dendritic as those of *dsu/dsu*, d^v/d^v mice, indicating that the level of coat color phenotype suppression can be directly correlated to the extent of restoration of normal melanocyte morphology. Recent studies (H. SWEET, personal communication) have also shown that the *ash* coat color is suppressed by *dsu*.

The three loci, *ln*, *ash* and *d*, have been classified previously into a gene family because they all produce a dilute coat color that appears causally associated with the presence of adendritic melanocytes. The findings presented here, indicating that all three mutations are at least partially suppressed by *dsu* and that these are indeed all separate loci, lend further support for this association. This association is also strengthened by genetic studies in progress in our laboratory that indicate that *dsu* is not capable of suppressing the coat color of several mutations effecting pigment deposition but not melanocyte morphology (K. J. MOORE, unpublished results).

The precise manner by which *dsu* suppresses mutant coat color phenotype is not known. However, it is likely that *dsu* is involved in enhancing the capacity of mutant melanocytes to make dendrites. A simple model is that *dsu* encodes a mutant protein that can partially compensate for the defect in *d*, *ln* and *ash* melanocytes or, alternatively, that suppression results from the abnormal temporal or developmental expression of an otherwise normal *dsu* product. These models are consistent with our two previous observations that *dsu* acts semidominantly and is capable of suppressing the dilute coat color phenotype of d^{120J}, a deletion mutation of the *d* locus (K. J. MOORE *et al.*, unpublished results). The three loci *ln*, *ash* and *d* may encode products involved in a single biochemical pathway or they may be members of a conserved gene family. That they are not involved in a single biochemical pathway is suggested by our observations that: (1) the dilute phenotype of *ash* d^v/*ash* d^v mice is somewhat lighter than *ash/ash* or d^v/d^v mice suggesting that the dilution of pigment produced by these two mutants is additive; and (2) the suppression of dilute coat color phenotype in *ash/ash* mice and *ln/ln* mice is somewhat reduced compared to the suppression of coat color phenotype in d^v/d^v mice.

In considering a model in which *d*, *ln* and *ash* (and possibly *dsu*) are members of a gene family, it is interesting to note that *d* and *ash* are tightly linked on chromosome *9* and that *ln* and *dsu* are loosely linked on chromosome *1*. It is also interesting to note that chromosomes *1*, *7* and *9* may represent paralagous chromosomes resulting from an ancient tetraploidization event that occurred before the radiation of the mammals (LUNDIN 1979). If true, then the genetic linkage data between *ln* and *dsu* as well as between *d* and *ash* presented here would seem to favor a hypothesis whereby a gene duplication event occurred before tetraploidization to produce *ash* and *d*, and possibly *ln* and *dsu*, with a subsequent chromosomal rearrangement(s) involving one or both pairs of loci.

An alternative explanation for the mechanism of action of *dsu* suggests that the wild-type product of *dsu* is involved in maintenance of the intercellular matrix and that the mutant form of *dsu* produces a cellular environment that is more conducive to dendritic growth of *d*, *ln* and *ash* melanocytes. This model is based upon a large body of data obtained from many laboratories concerning the mechanism of action of *d* and *ln*. For example, MARKERT and SILVERS (1956) have shown that melanocytes from d^v/d^v or *ln/ln* mice, when transplanted into the anterior chamber of the eye of mice that are wild-type at these loci, give rise to melanocytes that vary in morphology from adendritic to almost wild type. The authors concluded that *d* and *ln* exert their action within the melanocyte and that the capacity to produce dendrites is not absent in d^v/d^v or *ln/ln* melanocytes but is subdued in a compact tissue environment. Upon transfer into the less crowded cellular environment of the anterior chamber of the eye, the weak capacity to make dendrites is less inhibited and dendrites are produced.

Another series of experiments that is consistent with this hypothesis was done by QUEVEDO and MCTAGUE (1963). They exposed skin of d^v/d^v mice to UV irra-

diation and observed that the epidermal melanocytes of the treated mice developed more numerous and extensive dendrites than are present in untreated controls. However, they were not as developed as the dendrites found in untreated mice that were wild type at *d*. To account for this observation, they proposed that UV treatment might be increasing the permeability of the cellular interstices of the epidermis allowing dendrites to be expressed by d^v/d^v melanocytes. Finally, PRESTON *et al.* (1987) have reported that Cloudman melanoma cells in culture, which are genotypically d^v/d^v, can be induced to produce processes that resemble dendrites by treatment with α-melanocyte stimulating hormone. These studies suggest that d^v melanocytes are capable, but have a reduced capacity, to produce dendrites.

Ultimately, the mechanism of action of *dsu* should become clear once this gene is molecularly cloned and studied at the molecular and biochemical level.

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Note Added in Proof: We have recently produced mice that are homozygous for *dsu, ln, ash,* and d^v; they are light off-black in coat color.

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