A Noncoding Melanophilin Gene (*MLPH*) SNP at the Splice Donor of Exon 1 Represents a Candidate Causal Mutation for Coat Color Dilution in Dogs

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Abstract

Coat color dilution in several breeds of dog is characterized by a specific pigmentation phenotype and sometimes accompanied by hair loss and recurrent skin inflammation, the so-called color dilution alopecia or black hair follicular dysplasia. Coat color dilution (*d*) is inherited as a Mendelian autosomal recessive trait. In a previous study, *MLPH* polymorphisms showed perfect cosegregation with the dilute phenotype within breeds. However, different dilute haplotypes were found in different breeds, and no single polymorphism was identified in the coding sequence that was likely to be causative for the dilute phenotype. We resequenced the 5′-region of the canine *MLPH* gene and identified a strong candidate single nucleotide polymorphism within the nontranslated exon 1, which showed perfect association to the dilute phenotype in 65 dilute dogs from 7 different breeds. The A/G polymorphism is located at the last nucleotide of exon 1 and the mutant A-allele is predicted to reduce splicing efficiency 8-fold. An *MLPH* mRNA expression study using quantitative reverse transcriptase–polymerase chain reaction confirmed that *dd* animals had only about approximately 25% of the *MLPH* transcript compared with *DD* animals. These results provide preliminary evidence that the reported regulatory *MLPH* mutation might represent a causal mutation for coat color dilution in dogs.

Coat color dilution in dogs is characterized by a specific pigmentation phenotype and sometimes accompanied by hair loss and recurrent skin inflammation, the so-called color dilution alopecia (CDA; Kim et al. 2005) or black hair follicular dysplasia (BHFD; von Bomhard et al. 2006). Coat color dilution is inherited as a Mendelian autosomal recessive trait in various dog breeds (Schmutz et al. 1998).

The dilution phenotype occurs in different mammalian species and is characterized by a defective transport of melanosomes leading to large clumps of pigments within melanocytes, the so-called macromelanosomes. Three proteins have been described in humans and mice as being essential for even distribution, transport, and translocation of pigment granules. Myosin Va (MYO5A) has been observed to move melanosomes on actin filaments (Wu et al. 1998; Barral and Seabra 2004). RAB27A located on the surface of mature melanosomes, recruits melanophilin (MLPH) (Wu et al. 2002; Goud 2002), which acts as a linker protein between the me-

lanosome and the MYO5A-bound actin filament. All 3 genes have been found to be mutated in different mouse strains, and defects in these molecules give rise to lighter skin/coat color (Mercer et al. 1991; Wilson et al. 2000; Matesic et al. 2001). In humans, mutations in the orthologous genes have been demonstrated to cause the 3 types of Griscelli syndrome, a rare autosomal recessive disorder characterized by pigment dilution of the skin and hair (OMIM 214450; 607624; and 609227; Pastural et al. 1997; Menasche et al. 2000; Menasche et al. 2003). Recently, in cats, a single base pair deletion in exon 2 of the feline *MLPH* gene affecting both eumelanin and phaeomelanin pigment distribution was shown to cause the feline dilute coat color phenotype (Ishida et al. 2006).

In dogs, after initial mapping of the *MLPH* gene to CFA25q24 (Philipp, Quignon, et al. 2005), we reported a strong association for single-nucleotide polymorphisms (SNPs) in exon 2 of *MLPH* in dilute Doberman Pinschers,

Beagles, and Large Munsterlanders and in exon 7 in dilute German Pinschers (Philipp, Hamann, et al. 2005).

However, no causative mutations for the canine dilute phenotype have been identified in the *MLPH* gene so far. One possible explanation for the previously obtained results would be the existence of a regulatory *MLPH* mutation with effect on mRNA expression levels. To test this hypothesis, we analyzed about 11 kb of the 5′-region of the canine *MLPH* gene including the promoter region for dilute associated sequence polymorphisms in several breeds.

Materials and Methods

Mutation Analysis

DNA prepared from frozen ethylenediaminetetraacetic acid-stabilized blood samples of 285 purebred individuals from 7 dog populations segregating for the dilute coat color phenotype (131 German pinschers, 109 European Doberman Pinschers, 15 American Doberman Pinschers, 13 Rhodesian Ridgebacks, 7 Beagles, 6 Large Munsterlanders, and 3 Miniature Pinschers) and a single dilute and CDA-affected American Staffordshire was available for genotyping (Table 1). The coat color phenotype of the dogs was recorded based on their pedigree certificates and classified within the breeds as described (Philipp, Hamann, et al. 2005). The dilute animals were classified as homozygous for the recessive mutated dilute allele (*dd*) and the wild-type colored dogs were classified as carrying at least a single copy of the dominant wild-type allele (*D.*). Only those nondilute dogs where information about direct dilute colored offspring could be obtained were classified as obligate heterozygotes (*Dd*).

A contiguous 12564-bp sequence segment from the 5′-region of the canine *MLPH* gene including the promoter, the untranslated exon 1, and parts of intron 1 was used for polymerase chain reaction (PCR) primer design. A total of 15 primer pairs amplified 11905 bp (95%) of the 12564-bp interval between position 147996 and 160560 of EMBL entry BN000728 (Supplementary Table 1). For the resequencing, we used 12 dogs from 4 breeds: a single dilute Beagle, a single dilute and 2 nondilute European Doberman Pinschers, 2 dilute and 2 nondilute American Doberman Pinschers, and 2 dilute and 2 nondilute German Pinschers, respectively. The 6 nondilute dogs were assumed to be homozygous for the wild-type allele (*DD*) due to their previously established genotypes of *MLPH* exon 2 and 7 SNPs, respectively (Philipp, Hamann, et al. 2005).

PCRs were carried out using hotstart AmpliTaqGold (Applied Biosystems, Rotkreuz, Switzerland) at standard conditions at 60 °C annealing. The subsequent sequencing of the PCR products in both directions was performed after shrimp alkaline phosphatase (Roche, Basel, Switzerland) and exonuclease I (NEB, Axon lab, Baden, Switzerland) treatment using the PCR primers with the ABI BigDye Terminator Sequencing Kit 3.1 (Applied Biosystems) on an ABI 3730 capillary sequencer (Applied Biosystems). Some regions with extremely high GC content or long mononucleotide repeats comprising about 6% of the length of the amplified PCR

Table 1. Genotype frequencies of the *MLPH* exon 1 SNP in different dog breeds

Breed	Coat color	Dilute genotype	Number of animals	Exon 1 SNP (c.-22G>A)		
				AA	AG	GG
German Pinscher		Total	131			
	Dilute	*dd*	28	28		
	Wild type	*Dd*	10		10	
	Wild type	*D.*	93		37	56
Doberman Pinscher (European samples)		Total	109			
	Dilute	*dd*	13	13		
	Wild type	*Dd*	11		11	
	Wild type	*D.*	85		35	50
Doberman Pinscher (American samples)		Total	15			
	Dilute	*dd*	9	9		
	Wild type	*Dd*	2		2	
	Wild type	*D.*	4		1	3
Rhodesian Ridgeback		Total	13			
	Dilute	*dd*	7	7		
	Wild type	*Dd*	2		2	
	Wild type	*D.*	4		2	2
Beagle		Total	7			
	Dilute	*dd*	2	2		
	Wild type	*Dd*	4		4	
	Wild type	*D.*	1			1
Large Munsterlander		Total	6			
	Dilute	*dd*	2	2		
	Wild type	*Dd*	1		1	
	Wild type	*D.*	3		3	
Miniature Pinscher	Dilute	*dd*	3	3		
American Staffordshire	Dilute and CDA	*dd*	1	1		
Total			285	65	108	112

products could not successfully be resequenced (Supplementary Table 1). Haplotyping was performed using SAS/HAP-LOTYPE software, Version 2.1.3, of the SAS System for Windows (2003 SAS Institute Inc., Cary, NC).

In Silico Splice Site Analysis

To evaluate the possible functional consequences of polymorphisms in the vicinity of splice sites, we used the NNSPLICE 0.9 software (http://www.fruitfly.org/seq_tools/splice.html), which is based on a neural network to predict splice sites in genomic sequences (Reese et al. 1997). We also used a publicly available tool "Automated Splice Site Analyses" (https://splice.cmh.edu/) to predict the effect

of splice site mutations that is based on information theory (Nalla and Rogan 2005).

Quantitative Reverse Transcriptase–PCR

A total of 13 dogs, 3 dilute and 10 normal pigmented Beagle dogs, consisting of 3 juvenile littermate puppies and 10 adult unrelated dogs, were genotyped at the dilute coat color–linked *MLPH* exon 2 and exon 7 SNP markers and, subsequently, grouped into 3 different genotypes (3 *DD*, 7 *Dd*, 3 *dd*). From these dogs, RNA of a pigmented dorsal skin biopsy was isolated directly with the RNeasy 96 Universal Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The reverse transcription into cDNA was performed by using 200 U SuperScript III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany), an oligo-dT primer ($(T)_{24}V$), and 10 μl of the isolated RNA in a 20-μl reaction. A single PCR assay was used for quantification of the *MLPH* gene transcript (GenBank AJ920333) using a forward primer situated in exon 13 (5′-GGC CTA AGG ATC CGA ATG C-3′) and a reverse primer in exon 14 (5′-CGG AGA ATT ACT GAA CTT CCT TCT G-3′) amplifying an 80-bp product with a Taqman minor groove binding (MGB) probe located at the boundary of exon 13 and 14 (*MLPH* probe VIC labeled: 5′-CTT CGG ACG AGG TGA C-3′). Canine *GAPDH* transcript was determined as endogenous control using a forward primer situated in exon 4 (5′-GGC ACA GTC AAG GCT GAG AAC-3′) and a reverse primer in exon 5 (5′-CCA GCA TCA CCC CAT TTG AT-3′) amplifying a 101-bp product in combination with a FAM-labeled Taqman MGB probe spanning the boundary of exon 4 and 5 (5′-TCC AGG AGC GAG ATC-3′) according to the canine reference mRNA sequence (GenBank NM_001003142). The quantitative reverse transcriptase (qRT)-PCR was carried out with an ABI 7300 sequence detection system (Applied Biosystems) in a 20-μl reaction containing TaqMan Universal MasterMix (Applied Biosystems), 50 μM forward, 50 μM reverse primer, and 10 μM Taqman probe using an annealing and elongation temperature of 60 °C. The *MLPH* transcript–specific expression was normalized by the canine *GAPDH* expression level (ΔCt), and the relative expression level was calculated by the $2^{-\Delta\Delta Ct}$ method using the juvenile homozygous *DD* sample as calibrator (Livak and Schmittgen 2001). All assays were performed 3 times in duplicates.

Results and Discussion

In our previous study, we identified 3 different *MLPH* haplotypes that were associated with the dilute allele in different dog breeds. These 3 dilute haplotypes did not share any variant alleles in the coding region of the *MLPH* gene. However, we noted that the 3 different haplotypes had the same alleles at 3 SNPs in the 5′-region of the *MLPH* gene (Philipp, Hamann, et al. 2005).

Under the assumption that the dilute phenotype is caused by a single mutation in the studied breeds, we hypothesized

that a regulatory mutation in the 5′-region of the *MLPH* gene might be responsible for the dilute phenotype. If this hypothesis were true, then it would be expected that the causative mutation should be located in a region where the 3 different haplotypes share common alleles. Thus, it should be possible to fine map the position of the causative mutation by identifying the interval, where the 3 observed dilute haplotypes are identical. The boundaries of this interval would then correspond to old recombinations where the ancestral dilute chromosome recombined with wild-type chromosomes.

We chose 6 dilute and 6 wild-type colored dogs for the fine-mapping experiment. In the group of the dilute dogs, 2 dogs homozygous for each of the 3 previously observed dilute haplotypes were selected. We selected control dogs that were homozygous *DD* based on coding *MLPH* gene markers from the same breeds as the dilute dogs. In these 12 animals, we amplified and resequenced a 12.5-kb genomic segment comprising 9 kb of 5′-flanking sequence, exon 1, and 3 kb of intron 1 of the *MLPH* gene. PCR worked successfully for 11905 bp (95% of the entire interval); however, some of the 12 DNAs did not allow amplification of all 15 PCR products. Out of the 12564 targeted bases, 11161 bp (89%) could be accurately resequenced. The missing 11% were mainly due to an extreme GC content especially within the proximal promoter region of the *MLPH* gene (Figure 1; Supplementary Table 1).

Comparative sequencing revealed 73 polymorphisms consisting of 6 microsatellites, 4 indel polymorphisms, and 63 SNPs within this panel of dogs. The 9 deduced haplotypes for the examined animals are illustrated in abbreviated form in Figure 1 and completely listed in the Supplementary Table 2. Similar to our previous study, 3 different dilute associated haplotypes were observed. Two of these dilute haplotypes were exclusively found within Doberman Pinschers of American origin (*d2*) and German Pinschers (*d3*), respectively. The 3rd dilute haplotype (*d1*) was found in Beagles and Doberman Pinschers from European ancestry. This haplotype also occured in Large Munsterlander dogs (data not shown), and it probably represents the ancestral haplotype on which the hypothetical founder mutation occurred. The 3 different dilute haplotypes share a 7.8-kb segment of identical alleles, which presumably harbors the causative dilute mutation (Figure 1).

The comparison between the dilute associated haplotypes in this candidate interval and the 6 observed wild-type haplotypes revealed 14 polymorphic sites that were perfectly associated to the dilute allele (Figure 1, Supplementary Table 2). Among these associated polymorphisms, there are 11 SNPs, 2 indels, and 1 microsatellite. We ranked the likelihood of these polymorphisms to be causative based on their position within the *MLPH* gene. The most interesting among the 14 associated polymorphisms was an A/G SNP located at the last nucleotide of the untranslated exon 1 (c.-22G>A), and we speculated that it might have an effect on splicing efficiency (Figure 2).

Therefore, we genotyped all available 285 DNAs at this SNP marker and observed perfect association in 7 different
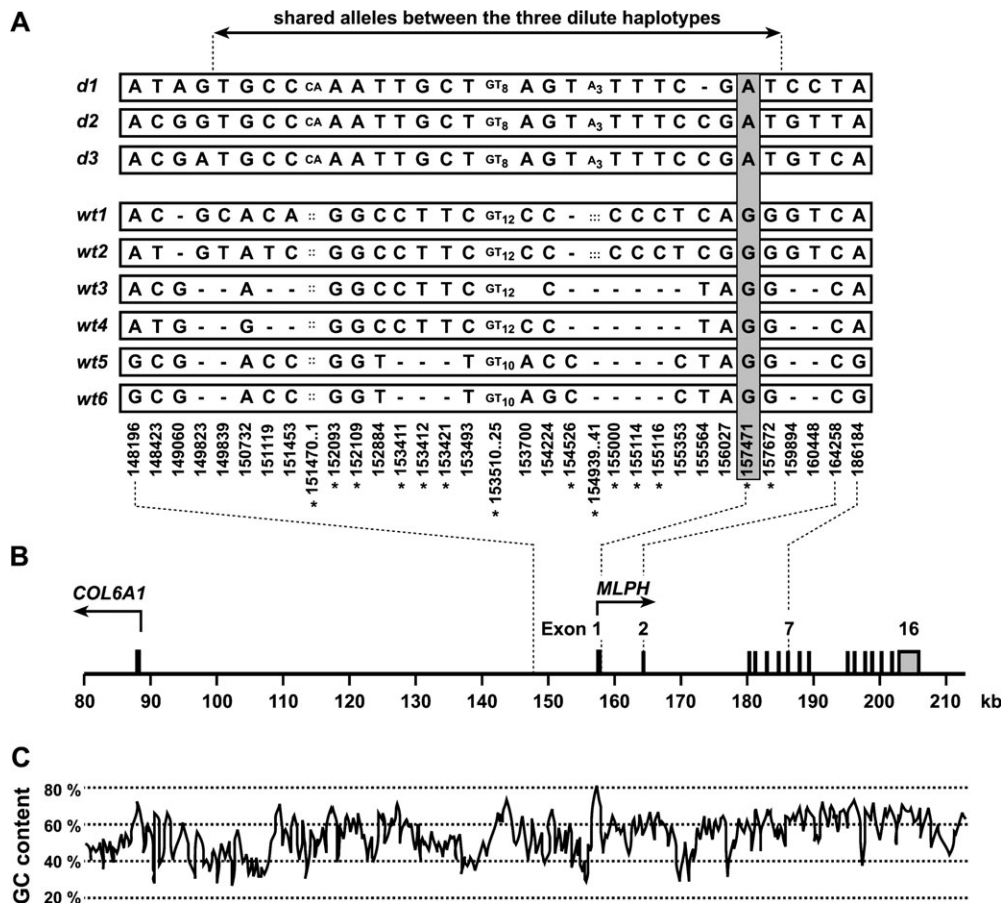
Figure 1. Fine mapping of the dilute mutation. (**A**) Overview of the dilute (*d1–d3*) and wild-type (*wt1–wt6*) haplotypes consisting of 33 selected polymorphisms within the canine *MLPH* gene. The 7.8-kb interval, where the 3 dilute haplotypes share identical alleles is indicated. Polymorphisms that are associated with the dilute phenotype in the 12 analyzed dogs are indicated with an asterisk. Note that we have omitted several additional polymorphisms that did not show a perfect association with the dilute phenotype. A complete list of all genotyping data is given in the Supplementary Table 2. (**B**) Genomic structure of the *MLPH* gene. The start codon is located in exon 2. (**C**) The calculated GC content of the reference sequence entry (EMBL BN000728) was calculated using a 300-bp window.

dog breeds (Table 1). In a previous study, we analyzed all coding regions of the canine *MLPH* gene to identify nucleotide polymorphisms within affected and healthy control dogs (Philipp, Hamann, et al. 2005). No single polymorphism was identified in the coding sequence or at splice sites that was likely to be causative for the dilute phenotype of all dogs examined. Within families, several of the discovered *MLPH* polymorphisms showed perfect cosegregation with the dilute phenotype. A synonymous *MLPH* SNP in exon 2 was proposed as diagnostic marker for dilute in several breeds; however, this marker was not informative in German Pinschers. In contrast, the newly identified exon 1 SNP marker shows perfect cosegregation with the dilute phenotype in several 2-generation families in 5 out of 7 examined breeds (Doberman Pinscher, German Pinscher, Beagle, Large Munsterlander, Rhodesian Ridgeback; data not shown). For 2 breeds (Miniature Pinscher and American Staffordshire), no family material was available.

One possible explanation for the effect of the observed exon 1 polymorphism would be a reduction of splicing efficiency. If the splicing of intron 1 is not correctly executed

Wildtype sequence:	C C $\underline{G}$ g t g a g
Mutated seq. (c.-22G>A):	C C $\underline{A}$ g t g a g
Consensus splice donor:	C_{38} A_{66} G_{84} g_{100} t_{100} a_{60} a_{76} g_{83}

Figure 2. Sequence at the 3′-end of exon 1 of the *MLPH* gene. The wild-type and mutated alleles, respectively, are shown. In the third line, the consensus sequence for human splice donors of the U2 GT-AG type is shown (Sheth et al. 2006). The indices in the consensus sequence describe the proportions of the respective conserved nucleotides in percent of the investigated 157 640 splice donor sites. Note that the c.-22G>A polymorphism affects a conserved nucleotide of the splice donor recognition motif.

by the cell, it seems likely that any aberrantly spliced transcripts would contain premature stop codons and be subject to nonsense-mediated decay. Using the splice site prediction program NNSPLICE 0.9, the wild-type sequence at the end of *MLPH* exon 1 represents a very efficient splice donor (score 0.99). According to this analysis, the mutant sequence is less efficient as splice donor (score 0.86). The analysis with a different and newer software confirmed this result. The "automated splice site analyses" predicted that the c.-22G>A polymorphism reduces U1 binding to the splice donor site by 8.3-fold to 12% of the wild-type level. Additionally, the wild-type sequence at the end of exon 1 represents a potential binding site for the accessory splicing factor SF2/ASF, which is completely abolished in the mutant sequence. Therefore, if the splicing of intron 1 of the *MLPH* gene should be dependent on SF2/ASF binding, then it would be expected that even less than 12% of the transcripts are spliced correctly.

If the *in silico* predicted effects are true, then we would expect a reduction in the steady-state *MLPH* mRNA levels in melanocytes as incorrectly spliced transcripts should be degraded by nonsense-mediated decay. To evaluate this hypothesis, skin biopsies were taken from pigmented skin of dilute and wild-type Beagles, and the *MLPH* expression levels were analyzed by qRT-PCR. In general, *DD* dogs showed the highest and homozygous dilute (*dd*) dogs showed the lowest *MLPH* expression level (Figure 3). Heterozygous *Dd* dogs were found at an intermediate level. There was considerable variation of the individual *MLPH* expression levels. Due to the limited number of samples, a meaningful statistical analysis was not possible. Juvenile dogs had a higher *MLPH* expression than adult dogs and were therefore analyzed separately. On average, *dd* animals had about 25% of the *MLPH* transcript compared with *DD* animals (Figure 3). The overall observed variability of expression levels between individuals might have been caused by differences in the cellular composition of the tissue samples as melanocytes constitute only a small fraction of skin tissue biopsies. The expression data corroborate the existing genetic data and provide the first functional evidence that a regulatory *MLPH* mutation might be causative for coat color dilution in dogs. At this time, it cannot be formally proved that the c.-22G>A mutation is a causative variant as we have not resequenced each nucleotide in the critical interval. Also we cannot exclude the possibility that any other of the 13 remaining polymorphisms that were in linkage disequilibrium with the c.-22G>A SNP and did not occur in the analyzed control dogs represents a causal mutation. However, the combination of genetic data, preliminary expression analysis, and the position within the gene, indeed, strongly argue for c.-22G>A being a causative mutation for coat color dilution in the investigated breeds.

Supplementary Materials

Supplememtary Tables 1 and 2 are available online at http://www.jhered.oxfordjounals.org/.
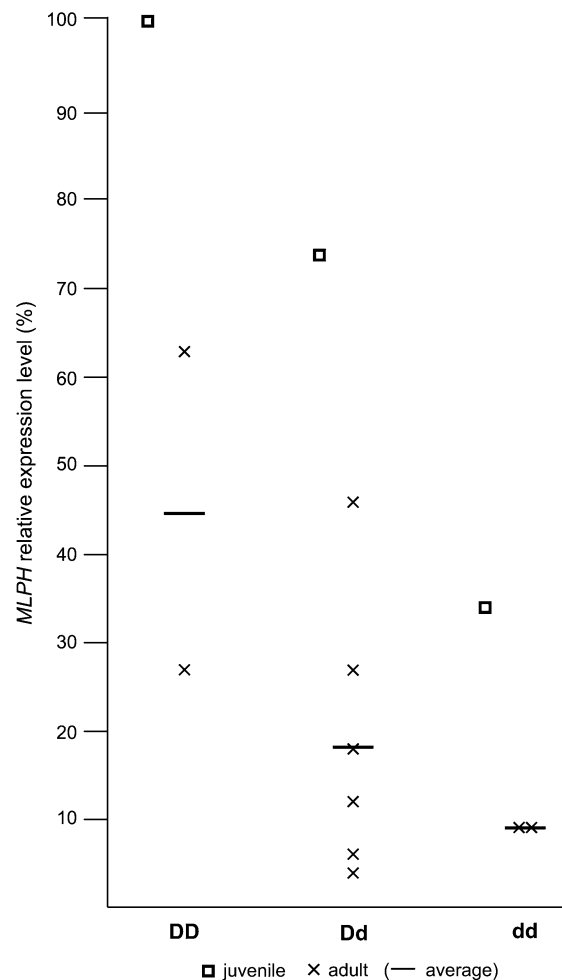

Figure 3. The *MLPH* expression level measured by quantitative real-time RT-PCR compared with the genotype and the age of the dog. Expression levels of adult dogs are represented by crosses, expression levels of juvenile dogs by open squares. Average values of the adult dogs are indicated. The highest observed relative *MLPH* expression level was arbitrarily set to 100%.

Acknowledgments

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