

The *Dominant white*, *Dun* and *Smoky* Color Variants in Chicken Are Associated With Insertion/Deletion Polymorphisms in the *PMEL17* Gene

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ABSTRACT

Dominant white, *Dun*, and *Smoky* are alleles at the *Dominant white* locus, which is one of the major loci affecting plumage color in the domestic chicken. Both *Dominant white* and *Dun* inhibit the expression of black eumelanin. *Smoky* arose in a White Leghorn homozygous for *Dominant white* and partially restores pigmentation. *PMEL17* encodes a melanocyte-specific protein and was identified as a positional candidate gene due to its role in the development of eumelanosomes. Linkage analysis of *PMEL17* and *Dominant white* using a red jungle fowl/White Leghorn intercross revealed no recombination between these loci. Sequence analysis showed that the *Dominant white* allele was exclusively associated with a 9-bp insertion in exon 10, leading to an insertion of three amino acids in the *PMEL17* transmembrane region. Similarly, a deletion of five amino acids in the transmembrane region occurs in the protein encoded by *Dun*. The *Smoky* allele shared the 9-bp insertion in exon 10 with *Dominant white*, as expected from its origin, but also had a deletion of 12 nucleotides in exon 6, eliminating four amino acids from the mature protein. These mutations are, together with the recessive *silver* mutation in the mouse, the only *PMEL17* mutations with phenotypic effects that have been described so far in any species.

DOMINANT *white* is one of the major loci influencing plumage color in chicken (SMYTH 1990). The *Dominant white* color was in fact one of the first traits to be investigated following Mendel's classical work (BATESON 1902) and the mutation was assigned the gene symbol *I* for its inhibiting effect on pigmentation (HURST 1905). *I* is incompletely dominant to the wild-type allele, *i*. *Dominant white* is a breed characteristic of White Leghorns giving the birds a pure white plumage without any patterns or markings (Figure 1). Beak and shanks are yellow and the eye is brown since *Dominant white* affects only melanocytes of neural crest origin (SMYTH 1990). A putative third allele at the *Dominant white* locus, denoted *Smoky* (*I***S*), has been identified. It arose in a White Leghorn line fixed for *Dominant white* and segregation data indicated that it is allelic to *I* (R. OKIMOTO,

B. PAYNE and D. SALTER, unpublished results). *Smoky* gives a grayish phenotype and is recessive to *Dominant white* but partially dominant to the wild-type allele (Figure 1). *Dun* (*I***D*) was identified as a spontaneous mutation in a Pit-gamecock bird (ZIEHL and HOLLANDER 1987). Segregation data indicated that *Dun* is allelic to *Dominant white*. *Dun* heterozygotes (*I***D*/*i*) show a *Dun* phenotype while homozygotes are whitish.

The *I* locus has been mapped to chicken linkage group E22C19W28 (RUYTER-SPIRA *et al.* 1996; SCHMID *et al.* 2000). We have recently confirmed this assignment by linkage analysis using our intercross between the red jungle fowl and the White Leghorn chicken (KERJE *et al.* 2003a). Three genes, *ERBB3*, *TUBAL1*, and *GLI*, have been mapped to the E22C19W28 linkage group and their homologs are all located on chromosome 12 in human and chromosome 10 in mouse (SCHMID *et al.* 2000). These chromosome regions in human and mouse harbor *Silver* encoding the melanocyte-specific *PMEL17* protein also denoted GP100. *PMEL17* is a type I integral membrane protein present in the melanosome and is a component of the fibrous striations upon which melamins are polymerized (BERSON *et al.* 2003; DU *et al.* 2003). *PMEL17* has a crucial role for the normal development

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AY636124–AY636129.

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FIGURE 1.—Birds expressing the wild-type, Dominant white, and Smoky phenotypes.

of eumelanosomes. It has been shown that the proteolytic cleavage and processing of *PMEL17* accompanies the restructuring of early eumelanosomes from amorphous rounded vesicles into elongated fibrillar structures (KUSHIMOTO *et al.* 2001). A single-base insertion at the 3'-end of *silver* (encoding *PMEL17*) in mouse results in a premature stop codon and a truncation of the last 25 amino acids in the carboxyterminal cytoplasmic region of the protein (MARTINEZ-ESPARZA *et al.* 1999; SOLANO *et al.* 2000). *silver* homozygotes show graying of the hair due to loss of follicular melanocytes (QUEVEDO *et al.* 1981). The truncated *PMEL17* protein was found in different fractions of homogenized mouse cells due to misrouting of the mutated protein. Thus, *PMEL17* was identified as a positional candidate gene for the Dominant white phenotype in chicken. (We have decided to denote this locus *PMEL17* and not *Silver*, as *Silver* is already used as the locus designation for another classical plumage color locus in the chicken.)

In this study we present strong evidence that *Dominant white*, *Dun*, and *Smoky* are all caused by mutations in *PMEL17*. Unique insertion/deletion polymorphisms (9–15 bp in length) were found in birds carrying these alleles.

MATERIALS AND METHODS

Animals: We have generated a resource pedigree for gene mapping by crossing one red jungle fowl male and three White Leghorn females. Four F_1 males and 37 F_1 females were intercrossed and ~1000 F_2 birds were hatched. The F_2 generation showed a considerable variation in plumage color and digital photos of 814 F_2 animals were taken and used for phenotypic classification (KERJE *et al.* 2003b). An F_3 generation was generated by intercrossing 50 females with 50 males from the F_2 population. A few F_3 individuals heterozygous at *PMEL17* were selected to produce 15 F_4 embryos for tissue sampling. Samples for DNA and RNA isolation were collected 14 days after fertilization of the eggs. The *PMEL17* genotypes of the embryos were determined by a DNA test.

DNA samples used for sequencing are listed in Table 5. Breeds denoted *b* are from University of Arkansas and the Broiler line is a selection line derived from White Plymouth Rock (DUNNINGTON and SIEGEL 1996).

Sequencing of genomic DNA and cDNA: Genomic DNA was isolated by standard methods, and primers to amplify the complete *PMEL17* gene were designed on the basis of a chicken cDNA sequence (GenBank D88348). The gene was sequenced in five parts using the primer pairs P1fwd/P1rev, P2fwd/P2rev, P3fwd/P3rev or P3.1fwd/P3.1rev, P4fwd/P4rev, and P5fwd/P5rev (for primer sequences see supplementary Table S1 at <http://www.genetics.org/supplemental/>); the P3 and P3.1 primers were used to amplify genomic DNA and cDNA, respectively.

The PCR for all primer pairs except P3 was performed in a total volume of 10 μ l containing ~50 ng DNA, 1 \times PCR buffer (QIAGEN, Valencia, CA), 2.5 mM $MgCl_2$, 200 μ M dNTPs, 2 pmol of each primer, and 0.5 units HotStarTaq polymerase (QIAGEN). The thermocycling included 10 min at 95°, followed by 50 cycles with 30 sec at 95°, 30 sec at 65°, and 2 min at 72°, ending with 10 min at 72°. The PCR with P3 primers was performed as described above but with 1 \times PCR buffer II (Applied Biosystems, Foster City, CA) and 0.5 units AmpliTaq-Gold (Applied Biosystems). The thermocycling included 4 min at 95°, followed by 45 cycles with 30 sec at 95°, 30 sec at 58°, and 2 min at 72°, ending with 10 min at 72°.

Most *PMEL17* fragments were difficult to amplify by PCR due to a very high GC content (68.4% average GC content for the entire gene). These fragments were therefore cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) prior to sequencing with the T7 and M13R universal primers. For those amplicons for which direct sequencing could be applied, the PCR primers were used for sequencing. The MegaBACE sequencing kit (Amersham Biosciences, Uppsala, Sweden) was used for sequencing and fragments were electrophoresed using the MegaBACE 1000 capillary instrument (Amersham Biosciences) and analyzed with the Sequence Analysis software (Amersham Biosciences). Sequences were controlled, aligned, and compared using the Sequencher 3.1.1 program (Gene Codes, Ann Arbor, MI).

***PMEL17* genotyping and linkage mapping:** *PMEL17* segregation was scored using the polymorphic repeat in exon 7. PCR amplification using the Pmel_exon7fwd and Pmel_exon7rev primers (Table S1 at <http://www.genetics.org/supplemental/>) gave 490- and 574-bp fragments associated with the *Dominant*

white and wild-type alleles, respectively. PCR was performed as described above in a total volume of 10 μ l with 0.5 units HotStarTaq DNA polymerase (QIAGEN). Fragments were separated using a MegaBACE 1000 capillary instrument (Amersham Biosciences) and analyzed with the Genetic Profiler software (Amersham Biosciences). Microsatellite marker *MCW317* was analyzed previously (KERJE *et al.* 2003a) and the same conditions were used for *MCW188* in the present study.

The *MYG1* cDNA sequence was available in a chicken EST database (pgr1n.pk003.120; <http://www.chickest.udel.edu>) and two single nucleotide polymorphisms (SNPs) were identified by partial sequencing of the gene. A fragment containing the SNPs was amplified by PCR with primers pyMYG1Bio and pyMYG1 (Table S1 at <http://www.genetics.org/supplemental/>) prior to pyrosequencing using the SNP reagent kit protocol (Pyrosequencing AB, Uppsala, Sweden). PCR was performed as described above with 0.5 units HotStarTaq DNA polymerase (QIAGEN) but with 1.75 mM MgCl₂ and 5 pmol of each primer. The pyrosequencing primer, pyMYG1seq (Table S1 at <http://www.genetics.org/supplemental/>), was designed to anneal just before the first SNP.

PMEL17, *MCW188*, and *MYG1* were mapped in relation to the other markers previously genotyped in the pedigree (KERJE *et al.* 2003a). The CRIMAP program (GREEN *et al.* 1990) and the functions BUILD and FLIPS were used to test the order of the markers. CHROMPIC was used to reveal unlikely recombination events.

The primer pair Pmel_exon10fwd and Pmel_exon10rev (Table S1 at <http://www.genetics.org/supplemental/>) was used for genotyping the insertion/deletion polymorphism in exon 10. The PCR was performed in a total volume of 10 μ l containing the same amount of reagents as for the PCR using genomic DNA as described above. The thermocycling included 10 min at 95°, followed by 50 cycles with 30 sec at 95°, 30 sec at 60°, and 45 sec at 72°, ending with 5 min at 72°. Fragments were separated using a MegaBACE 1000 capillary instrument (Amersham Biosciences) and analyzed with the Genetic Profiler software (Amersham Biosciences).

Expression analyses: Total RNA from 14-day-old whole embryos was isolated with the RNeasy mini kit according to the protocol for animal tissues (QIAGEN). The quality and concentration were checked with the Bioanalyzer (Agilent Technologies, Palo Alto, CA). Total RNA was treated with DNase according to the instructions for the DNA-free kit (Ambion, Austin, TX) prior to cDNA synthesis using the First-Strand cDNA Synthesis kit (Amersham Biosciences). cDNA and genomic DNA from *PMEL17* heterozygotes were used for allele quantification with pyrosequencing. A 164-bp fragment containing a G/A SNP at nucleotide position 2184 in the gene was amplified using PCR with primers pyPmelBio and pyPmelrev (Table S1 at <http://www.genetics.org/supplemental/>). The PCR was performed in a total volume of 15 μ l including ~20 ng cDNA, 1 \times PCR Buffer (QIAGEN), 2.5 mM MgCl₂, 200 μ M dNTPs, 3 pmol of each primer, and 0.75 units HotStarTaq polymerase (QIAGEN) and the following thermocycling was applied: 10 min at 95°, 45 cycles of 30 sec at 95°, 30 sec at 61°, and 30 sec at 72°, ending with 5 min at 72°. The sequencing primer, pyPmelseq (Table S1 at <http://www.genetics.org/supplemental/>) was designed to anneal just in front of the SNP. The difference in expression between the two alleles was determined by the peak heights in the pyrogram.

In vitro expression of the PMEL17 transmembrane region:

The so-called S-segment engineered into the *lep* gene in the pGEM1-based plasmid 67 (HESSA *et al.* 2003) was replaced with oligonucleotides encoding the predicted transmembrane domain of *PMEL17* from wild type (WT; codons 707–749), *Dominant white* (WAP; codons 679–724), and *Dun* (codons

677–714) alleles. Each transmembrane segment of *PMEL17* was generated by two sequential annealing reactions followed by a ligation reaction. First, six different annealing reactions composed of 13.6 μ M of 5' phosphorylated, complementing primer pairs were set up (for primer sequences see Table S1 at <http://www.genetics.org/supplemental/>). The annealing buffer was 20 mM Tris-HCl, pH 7.4, 2 mM MgCl₂, and 50 mM NaCl. The reaction was incubated at 85° for 10 min and slowly cooled down to 30°. Second, equal amounts of annealed primers from the first annealing reaction were mixed together (for WT, annealing reactions 1, 2, and 3; for WAP, annealing reactions 1, 4, and 6; and for Dun, annealing reactions 1, 2, and 5) and incubated at 65° for 5 min and slowly cooled down to 30°. The ligation reaction was prepared by adding the annealed product, *SpeI*- and *KpnI*-digested plasmid 67, ligation buffer, and T4 ligase (Promega, Madison, WI). The reaction was incubated at room temperature overnight. After *Escherichia coli* transformation and plasmid isolation, each construct was confirmed by DNA sequencing.

In vitro transcription and translation was performed as in HESSA *et al.* (2003), using 10 μ l SP6 express TnT reaction mix (Promega), 0.5 μ g of plasmid, 7.5 μ Ci ³⁵S (Amersham Bioscience), and 1 μ l (4 eq) of canine pancreatic microsomes (a gift from Masao Sakaguchi, Fukuoka, Japan), and incubating at 30° for 90 min. Translation products were analyzed by SDS-PAGE and the gel was imaged on a Fuji FLA-3000 phosphorimager (Fuji Instruments).

RESULTS

Inheritance of plumage colors: The segregation at the *Dominant white* (*I*), *Extended black* (*E*), *Barred* (*B*), and *Silver* (*S*) loci among the ~800 F₂ birds in our red jungle fowl \times White Leghorn intercross has recently been reported (KERJE *et al.* 2003b). The segregation at the *I* locus did not deviate significantly from the expected 3:1 ratio, indicating that it is epistatic to the other color loci. A sex difference in plumage color due to the segregation at the sex-linked *Silver* and *Barred* loci was noted.

Linkage analysis: The P3 primers were used to amplify a part of *PMEL17* exon 7 from genomic DNA. Sequencing of PCR products revealed a difference in the number of a 72-bp repeat between the red jungle fowl and the White Leghorn. This polymorphism was used to map *PMEL17* in relation to the *Dominant white* locus. The segregation at *PMEL17* in the F₂ generation did not deviate significantly from the expected 1:2:1 ratio ($\chi^2 = 1.49$, d.f. = 2, $P = 0.47$; see Table 2 for actual segregation data).

The linkage analysis included two loci, *MCW317* and *I*, already typed in the pedigree, and three additional markers, *MCW188*, *MYG1*, and *PMEL17*; *MYG1* was included to improve the comparative map for linkage group E22C19W28. All pairwise two-point LOD scores among these five loci were highly significant (Table 1). The two-point analysis did not indicate any recombination between *Dominant white* and *PMEL17* among 773 informative offspring (Table 1). However, a closer examination of the association between *PMEL17* genotypes and plumage color revealed three putative recom-

TABLE 1

Two-point linkage analysis of *Dominant white* (*I*), *PMEL17*, and marker loci on chicken linkage group E22C19W28

Marker pair		θ	Z
<i>I</i>	<i>PMEL17</i>	0	107.2
<i>I</i>	<i>MYG1</i>	0.13	26.4
<i>I</i>	<i>MCW188</i>	0.09	32.8
<i>I</i>	<i>MCW317</i>	0.21	6.7
<i>PMEL17</i>	<i>MYG1</i>	0.12	75.9
<i>PMEL17</i>	<i>MCW188</i>	0.10	70.8
<i>PMEL17</i>	<i>MCW317</i>	0.28	8.3
<i>MYG1</i>	<i>MCW188</i>	0.14	41.8
<i>MYG1</i>	<i>MCW317</i>	0.14	24.7
<i>MCW188</i>	<i>MCW317</i>	0.24	7.9

θ , recombination fraction; Z, LOD score.

binants (Table 2). Three individuals classified as white, white with black spots, or cream (all assumed to carry the *Dominant white* allele) were scored as homozygous for the *PMEL17* allele inherited from the red jungle fowl. However, CHROMPIC analysis revealed that these three animals (representing three different full-sib families) appeared as unlikely double recombinants. This implies that these three discrepancies from a complete association between *PMEL17* and presumed *I* genotypes do not reflect true recombinants. The deviations may be due to a few genotype/phenotype mismatches in this large sample but it is also possible that a combination of genotypes at other loci affecting plumage color in rare cases may mimic the effect of the *Dominant white* allele. These three individuals were excluded from further analysis.

Multipoint analysis revealed the following map order and sex-averaged map distances (in Kosambi centimorgans) for this linkage group: *MCW317*—19.5—*MYG1*—12.8—*I/PMEL17*—11.5—*MCW188* with no recombination between *PMEL17* and *I*. This order was supported by a LOD score >6 compared with all other possible orders.

Association between *PMEL17* genotypes and plumage color: The very close linkage between *PMEL17* and *Dominant white* allowed us to investigate the association between *PMEL17/Dominant white* genotypes and plumage color (Table 2). There was a marked sexual dimorphism for plumage color in this pedigree as reported in our previous study of the association between *MCIR* genotypes and variation in plumage color (KERJE *et al.* 2003b). Therefore, the data are presented separately for each sex. However, all individuals, irrespective of sex and scored as colored, were homozygous (*J/J*) for the jungle fowl allele at *PMEL17* (Table 2). More than 90% of the females scored as *PMEL17* *W/W* were purely white whereas only ~60% of the heterozygotes (*J/W*) were purely white. The *Dominant white* allele appeared

TABLE 2

Plumage color and *PMEL17* genotype distributions in the F₂ generation of a White Leghorn/red jungle fowl intercross

Phenotype class	<i>I</i> genotype ^a	<i>PMEL17</i>			Total
		<i>W/W</i>	<i>J/W</i>	<i>J/J</i>	
Females					
White	<i>I/-</i>	91	120	1 ^d	212
Cream ^b	<i>I/-</i>	5	35	1 ^d	41
White with black spots	<i>I/-</i>	0	33	1 ^d	34
Colored ^c	<i>i/i</i>	0	0	103	103
Total for females		96	188	106	390
Males					
White	<i>I/-</i>	32	46	0	78
Cream ^b	<i>I/-</i>	12	13	0	25
White with red/brown	<i>I/-</i>	46	155	0	201
White with black spots	<i>I/-</i>	0	2	0	2
Colored ^c	<i>i/i</i>	0	0	77	77
Total for males		90	216	77	383
Total across sex		186	404	183	773

J, allele inherited from red jungle fowl; *W*, allele inherited from White Leghorn.

^a Presumed genotype at the *Dominant white* (*I*) locus.

^b Cream is a pale red/yellow phenotype with no black pigment (see KERJE *et al.* 2003b).

^c This group includes birds that were black, gray, wild type and barred (KERJE *et al.* 2003b).

^d These animals appeared as unlikely double recombinants in the linkage analysis.

less dominant in males since only ~30% of the animals scored as *PMEL17* *W/W* and only ~20% of the *J/W* heterozygotes were purely white. This sex difference is at least partially explained by the different genotype distributions at the sex-linked *Barred* and *Silver* loci. Fifty percent of the female F₂ birds were hemizygous for these dominant alleles inherited from the White Leghorn F₀ females. In males, 50% of the F₂ animals were heterozygous for *Barred* and *Silver*, but none were homozygous, and it is well known that males that are heterozygous at these loci are less white than homozygotes.

Some phenotypes, such as white with black spots, were almost entirely composed of heterozygous animals. Most animals that scored as partially white expressed red colors.

Sequence analysis of *PMEL17* using genomic DNA: Sequencing of *PMEL17* using genomic DNA from birds carrying the wild-type, *Dominant white*, *Dun*, or *Smoky* allele revealed a considerable amount of polymorphism (Table 3). The sequenced region started 32 bp before the start codon and included 111 bp of the 3' UTR. Exon and intron borders were determined by comparing genomic and cDNA sequences obtained in this study with the chicken *PMEL17* cDNA sequence D88348 and the EST sequences BU217288 and BU315027 (all from GenBank). The comparison revealed that the chicken

TABLE 3
DNA sequence polymorphism in PMEL17 among chicken breeds

Exon/intron	Nucleotide position	Red jungle fowl (<i>i/i</i>)	Black Langshan (<i>i/i</i>)	Broiler line (<i>i/i</i>)	White Leghorn (<i>I/I</i>)	Smoky (<i>S/S</i>)	Dun (<i>D/D</i>)	
Intron 1	116	C	T					
	120	A	G				G	
	172	T	C	Y			C	
	189	C		Y				
	195	T	C				C	
	209	G					A	
	211	G	T				T	
	305	A	G				G	
	311	C	A				A	
	356	G	C	S			C	
	388	T					C	
	454	C	T					
	Exon 2	676	T	C	Y			
680		C					T	
Intron 2	798	C	G	G			G	
Exon 3	905	C	T	Y				
	942	G					A	
Intron 3	1033	A					T	
	1035	A					G	
	1041	A					G	
	1043	G					A	
	1045	T					A	
	1047	G					A	
	1065	G					A	
	1079				D ₁₂	D ₁₂	D ₁₂	D ₁₂
Intron 4	1291	A	G	G				
	1315	C		G			G	
Intron 5	1569		D ₂₂	D ₄₄ ^a	D ₂₂	D ₂₂	D ₄₄	
	1575	G	A		A	A		
Exon 6	1626	C		T				
	1684	C		T				
	1793	C	T	T				
	1811	G			A	A		
	1828					D ₁₂		
	1872	C		Y				
	2102	C	T					
	2184	A	G		G	G		
Intron 6	2312	C		T				
	2369	T					G	
Exon 7	2464	C	T					
	2473	A	G	G	G	G		
	2489	r ₄	r ₃	r ₃	r ₃	r ₃		
	2864	G		A				
Intron 7	2982	A	G					
	2983	G	C					
	2993	G	A					
Exon 8	3072	G	A					
Intron 8	3236	G		A				
	3246	A	G	G				
	3270		I ₄	I ₄				
	3289	C	T					
	3292	A	C	C				
	3308	C	T	T				
	3329	* ₄	G+* ₂	G+* ₂				
	Exon 9	3346	G	C	C			
		3370	G		A			
		3373	C		T			

(continued)

TABLE 3
(Continued)

Exon/intron	Nucleotide position	Red jungle fowl (<i>i/i</i>)	Black Langshan (<i>i/i</i>)	Broiler line (<i>i/i</i>)	White Leghorn (<i>I/I</i>)	<i>Smoky</i> (<i>S/S</i>)	<i>Dun</i> (<i>D/D</i>)
Intron 9	3561	G		C			
	3564	T		A			
	3582	T		G			
Exon 10	3671				I ₉	I ₉	
	3695						D ₁₅
Intron 10	3741	T	C	Y			
Exon 11	3819	C					T

The *Dominant white* genotypes are given in parentheses. A blank space indicates identity to the master sequence (red jungle fowl). Heterozygous positions: S, G/C; Y, C/T. Length polymorphisms: D_x, deletion; x, number of deleted bases; I_x, insertion; x, number of inserted bases; r_x, 72-bp repeat; x, number of repeats; *_x, CA repeat.

^aThis animal was heterozygous for the deletion.

gene has 11 exons like human *Silver* (PMEL17) but the gene is only 4.1 kb compared to 11.8 kb in human (<http://www.ncbi.nlm.nih.gov/LocusLink/>) due to smaller introns. We noted that the chicken D88348 GenBank sequence (MOCHII *et al.* 1991) contains a likely sequencing error since it is missing the two last nucleotides in exon 8, which leads to a frameshift. We are convinced that the sequence reported in this article is correct since it was confirmed using both genomic DNA and cDNA, and it gives a better alignment with the corresponding mammalian homologs. The last 377 nucleotides of EST BU217288 do not show any similarity with any of the other chicken *PMEL17* sequences and probably represent a cloning artifact (data not shown).

We identified a total of 56 SNPs and eight insertion/deletion polymorphisms across populations (Table 3).

There was no sequence difference between the White Leghorn lines L13 from Sweden and ADOL from the United States. The White Leghorn and the red jungle fowl sequences differed from each other at six positions but only two of these were uniquely associated with the *Dominant white* allele, a synonymous substitution at position 1836 in exon 6 and a 9-bp insertion in exon 10. The *Smoky* allele was identical to *Dominant white* with the exception of a unique 12-bp deletion in exon 6. *Dun* was associated with a *PMEL17* sequence that was clearly distinct from *Dominant white* and that possessed 13 unique SNP alleles and a unique 15-bp deletion in exon 10.

The variable positions at the amino acid level are shown in Table 4. Seven missense mutations were observed in total but none was exclusively associated with

TABLE 4
Amino acid polymorphisms in the chicken *PMEL17* protein

Breed	Composition of repeats										
	34	35	105	232	280-4	399	Repeat	586	723-5	731-5	740
Repeat type A	TAGATDGDVAVGPTAAATAESIADP										
Repeat type B	----- -----										
Repeat type C	IV -----										
	Amino acid residue										
Red jungle fowl (<i>i/i</i>)	S	A	G	A	PTVT	N	A-A-A-C	E		LGTA	R
Black Langshan (<i>i/i</i>)	P	-	-	-	-	D	A-B-A	-	-	-	-
Broiler line (<i>i/i</i>)	-	-	-	V	-	D	A-A-C	K	-	-	-
White Leghorn (<i>I/I</i>)	-	-	-	-	-	D	A-B-A	-	WAP	-	-
<i>Smoky</i> (<i>S/S</i>)	-	-	-	-	del	D	A-B-A	-	WAP	-	-
<i>Dun</i> (<i>D/D</i>)	-	V	S	-	-	-	-	-	-	del	C

(Top) Alignment of the corresponding amino acid sequences for the three different forms of the 72-bp repeat occurring in chicken *PMEL17* exon 7. (Bottom) Polymorphism among six chicken breeds (the *Dominant white* genotypes are given in parentheses). A dash indicates identity to the master sequence and dots indicate deletions.

the *Dominant white* or *Smoky* alleles. Three missense mutations were found only in the *Dun* allele. The repeat comprising 24 amino acids showed both sequence variation and variation in the number of repeated copies (Table 4). The polymorphic nature of this repeated region does not appear functionally important since there was no correlation between repeat-type composition and effect on plumage color. The 9-bp insertion, exclusively associated with *Dominant white* and *Smoky*, resulted in the insertion of the amino acid triplet WAP in the transmembrane region. Interestingly, the 15-bp deletion associated with *Dun* also alters the transmembrane region and leads to a deletion of five amino acids. The unique 12-bp deletion in exon 6, associated with *Smoky*, causes a deletion of four amino acids.

An alignment of the chicken sequence with the homologous sequences in humans and mouse shows that some parts of the PMEL17 protein are well conserved between birds and mammals whereas other parts are not, and these are hard to align (Figure 2). The proteolytic cleavage site dividing PMEL17 into an α - and β -chain (BERSON *et al.* 2003) is well conserved between species. The *Dominant white*, *Dun*, and *Smoky* insertion/deletions all disrupt fairly well-conserved regions of PMEL17.

Sequence polymorphisms across breeds: The polymorphic 72-bp repeat in exon 7 and the insertion/deletion polymorphisms in exon 10 associated with *Dominant white* and *Dun* were screened across breeds (Table 5). A surprisingly high degree of variation in the number of repeats was found, ranging from one repeat in the Fayoumi to four in several breeds. It is also clear that *Dominant white* has the same composition of repeats, two A and one B repeat, as do Black Langshan and Rhode Island Red that carry the recessive *i* allele. This finding excludes the repeat region from being causative for the Dominant white phenotype at least on its own. In contrast, the insertion in exon 10 was found only in the three different breeds carrying *Dominant white* (one broiler and two White Leghorn lines) and in the *Smoky* allele assumed to be derived from *Dominant white*. The 15-bp deletion in exon 10 was found associated only with the *Dun* allele.

Expression analysis of PMEL17: Total RNA was isolated from 2-week embryos from the F₄ generation with the genotypes *I/I*, *I/i*, and *i/i*. Sequence analysis of the entire PMEL17 coding sequence from the two homozygotes (*I/I* and *i/i*) showed that both transcripts were expressed and the sequence differences detected using genomic DNA were confirmed. No other sequence difference or variant transcripts were detected.

To investigate a possible difference in the level of PMEL17 expression associated with the *I* and *i* alleles, we took advantage of the synonymous SNP at nucleotide position 2184 in exon 6 that distinguishes the two alleles in our pedigree (Table 3). The relative expression of

the two alleles in nine heterozygous individuals was quantified by pyrosequencing of RT-PCR products. The quantitative analysis of the peak heights indicated an 8% higher expression of the *Dominant white* allele whereas control reactions using genomic DNA from the same animals did not indicate a significant difference in amplification efficiency between alleles. However, it is very unlikely that this minor difference in expression level can cause the dramatic phenotypic difference observed between Dominant white and wild-type chicken (Figure 1).

Membrane insertion of mutant PMEL17 transmembrane segments: To test whether the observed insertion/deletion polymorphisms in PMEL17 exon 10 of *Dominant white* and *Dun* disrupted the formation of the transmembrane helix, insertion into ER-derived rough microsomes was analyzed by *in vitro* transcription/translation (Figure 3). Briefly, the transmembrane segment of PMEL17 (denoted PMEL in Figure 3A) was inserted into the luminal P2 domain of the well-characterized integral membrane protein leader peptidase (Lep), where it is flanked by two Asn-X-Thr acceptor sites for N-linked glycosylation (G1, G2). The P2 domain is efficiently translocated into the lumen of the microsomes when wild-type Lep is translated in the presence of rough microsomes (JOHANSSON *et al.* 1993). If the PMEL17-derived segment forms a transmembrane helix that is inserted into the microsomal membrane, only the G1 site is glycosylated by the lumenally disposed oligosaccharyl transferase (Figure 3A, top left), whereas both the G1 and the G2 sites are modified if the PMEL17-derived segment fails to form a transmembrane helix (Figure 3A, top right; *cf.* SÄÄF *et al.* 1998).

As shown in Figure 3B, the PMEL17-derived segments representing all three alleles (wild type, *Dominant white*, and *Dun*) were able to form transmembrane segments since the predominant form of the Lep-PMEL17 protein was glycosylated on only one site. For the *Dun* allele, a small fraction of the molecules was glycosylated on both sites, indicating that this variant is near the threshold where the formation of the transmembrane helix will be compromised.

DISCUSSION

This study has shown that mutations in PMEL17 are causing the Dominant white, Dun, and Smoky plumage phenotypes in chicken. This conclusion is supported by a number of facts. All colored birds among the ~800 F₂ progeny in our red jungle fowl/White Leghorn intercross were homozygous for the PMEL17 allele inherited from the red jungle fowl. All F₂ birds expressing any black pigment carried at least one copy of the wild-type PMEL17 allele. Furthermore, an identical PMEL17 sequence was associated with *Dominant white* in two different White Leghorn lines and a broiler line. The lack of convincing recombinants among ~800 F₂ birds and

	Signal sequence									
chicken_i	MRLHGAI ¹ VLL	AALLALVTAQ	QRGGGRSRGG	VKGS ² AWGGRP	APFRS ³ WDTAR	YRPWQEGTAR	QNDCWRGGDV	TFDISNDAPT		
chicken_I	-----	-----	-----	-----	-----	-----	-----	-----		
chicken_S	-----	-----	-----	-----	-----	-----	-----	-----		
chicken_D	-----	-----	-----	-----	-----	-----	-----	-----		
human	MDLVLKRC	LLH--VIG-L	LAV-ATKVPR	NQDWLGV ⁴ S-Q	LRTKA-NRQL	-PE-T-. . .Q	RL-----Q-	SLKV---G--		
mouse	M-VQRRS	FLPVLVLS-L	LAV-ALEGRS	NQDWLGV ⁵ P-Q	LVTKT-NRQL	-PE-T-. . .VQ	GSN-----Q-	SLRVI---G--		
mouse_silv	M-VQRRS	FLPVLVLS-L	LAV-ALEGRS	NQDWLGV ⁶ P-Q	LVTKT-NRQL	-PE-T-. . .VQ	GSN-----Q-	SLRVI---G--		
chicken_i	LVGARATFSI	ALRFPGTQTV	LPDGRVVWSQ	NCTVNGTRML	QGD ⁷ PVYPEQL	AEGSDGVFPD	GQPFPRSAWG	KGRFVYVWVW		
chicken_I	-----	-----	-----	-----	-----	-----	-----	-----		
chicken_S	-----	-----	-----	-----	-----	-----	-----	-----		
chicken_D	-----	-----	-----	-----	-----	-----	-----	-----		
human	-I--N-S---	--N--S-K-	---Q-I-VN	-TII--SQVW	G-Q---QET	DDAC. . I---	-G-C-SGS-S	QKRS-----K		
mouse	---N-S---	--H--S-K-	---Q-I-AN	-TII--SQVW	G-Q---QEP	DDAC. . ----	-G-C-SGPKP	PKRS-----K		
mouse_silv	---N-S---	--H--S-K-	---Q-I-AN	-TII--SQVW	G-Q---QEP	DDAC. . ----	-G-C-SGPKP	PKRS-----K		
chicken_i	TWGRYQVVD	GATSQ ⁸ LTVGT	DGVALGSYTM	EVVVYHYRGR	QRFIPIGHAS	TQFSITDQVP	IAVDVTQLEV	AAGDGGSFVR		
chicken_I	-----	-----	-----	-----	-----	-----	-----	-----		
chicken_S	-----	-----	-----	-----	-----	-----	-----	-----		
chicken_D	-----	-----	-----	-----	-----	-----	-----	-----		
human	---Q---LG	-PV-G-SI--	GRAM--TH--	--T--R--S	RSYV-LA-S-	SA-T-----	FS-S-S--RA	LD-GNKH-L-		
mouse	---K---LG	-PV-RSSIA-	GHAK--TH--	--T--R--S	-SYV-LA--	ST-T-----	FS-S-S--QA	LD-ETKH-L-		
mouse_silv	---K---LG	-PV-R-SIA-	GHAK--TH--	--T--R--S	-SYV-LA--	ST-T-----	FS-S-S--QA	LD-ETKH-L-		
chicken_i	NRPVAFNVRL	HDP ⁹ SHYLRDA	DISYSWDFGD	QSGTLISRSP	TVTHTYLQAG	SFAARLVLQA	AIPLSSCGTS	APPVVDPTTG		
chicken_I	-----	-----	-----	-----	-----	-----	-----	-----		
chicken_S	-----	-----	-----	-----	-----	-----	-----	-----		
chicken_D	-----	-----	-----	-----	-----	-----	-----	-----		
human	-Q-LT-ALQ-	---G--AE-	-L--T-----	S-----AL	V-----EP-	PVT-QV----	---T--S-	PV-.....		
mouse	-H-LI-ALQ-	---G--AE-	-L--T-----	GT-----AL	D-----ES-	-VT-QV----	---V--S-	PV-.....		
mouse_silv	-H-LI-ALQ-	---G--AE-	-L--T-----	GT-----AL	D-----ES-	-VT-QV----	---V--S-	PV-.....		
chicken_i	PVPSLGPTAT	QPVGPTGSGT	ATAPSNLTGS	GTAAPGTTA	APRASGAPAE	PTGVSVAVLS	DSAATEPLPD	PVLSTAVANA		
chicken_I	-----	-----	-----	-----	-----	-----	-----	-----		
chicken_S	-----	-----	-----	-----	-----	-----	-----	-----		
chicken_D	-----	-----	-----	-----	-----	-----	-----	-----		
human-T-DGHR	P--EAPN-TA	-QVPTTEVVG	TTPGQAPT--	-S-TTSVQVP	TTEVI ¹⁰ STA-V	QMPTAESTGM		
mouse-T-DGYM	P--EAPG-T-	RQGT ¹¹ TTKVVG	TTPGQMPTTQ	-S-TT-VQMP	TTEV-ATTSE	QM-T.....		
mouse_silv-T-DGYM	P--EAPG-T-	RQGT ¹² TTKVVG	TTPGQMPTTQ	-S-TT-VQMP	TTEV-ATTSE	QM-T.....		
chicken_i	AAGTDPTADP	LPPTSVSSGG	DAPGTVAPTA	VEGSVAAGVG	TAEDVAAATP	GATAADVAVD	Repeat 1 TAGATDGDV	GPTAAATAES		
chicken_I	-----	-----	-----	-----	-----	-----	-----	-----		
chicken_S	-----	-----	-----	-----	-----	-----	-----	-----		
chicken_D	-----	-----	-----	-----	-----	-----	-----	-----		
human	TPEKV-VSEV	MGT-LAEMST	PEATGMT-AE	-SIV-LS-TT	A-QVTTTEWV	ET--RELPIP	EPEGP-ASSI	MS-ESI-GSL		
mouseSAV	IDT-LAEVST	TEGTGTT--RPS-TT	V-QATTTE..GP-ASPL	L--QSS-GSI		
mouse_silvSAV	INT-LAEVST	TEGTGTT--RPS-TT	V-QATTTE..GP-ASPL	L--QSS-GSI		
chicken_i	Repeat 2 IADPTAGATD	Repeat 3 GDAVGPTAAA	Repeat 4 TAESIADPTA	GATDGDVAVP	TAAATAESIA	DPIVGATDGD	AVGPTAAATA	ESIADPTAGA		
chicken_I	-----	-----	-----	-----	-----	-----	-----	-----		
chicken_S	-----	-----	-----	-----	-----	-----	-----	-----		
chicken_D	-----	-----	-----	-----	-----	-----	-----	-----		
human	GPLLDGT--		
mouse	SPLDDTD--		
mouse_silv	SPLDDTD--		
chicken_i	TAVSSGSATA	GATAEPLLLV	K ¹³ QOPEAEPT	GCVLYRYGTF	STELNIVQGI	ESVAIVQVVP	AAPEGSGNSV	ELTVTCEGSL		
chicken_I	-----	-----	-----	-----	-----	-----	-----	-----		
chicken_S	-----	-----	-----	-----	-----	-----	-----	-----		
chicken_D	-----	-----	-----	-----	-----	-----	-----	-----		
human-R--	--V-LD...-S-	-VT-D----	--AE-L-A--	S...-E-DAF	---S-Q-G-		
mouseIM--	--V-LD...-S-	-LA-D----	--AE-L-A--	F...SE-DAF	---S-Q-G-		
mouse_silvIM--	--V-LD...-S-	-LA-D----	--AE-L-A--	S...SE-DAF	---S-Q-G-		
chicken_i	PEEVCTV ¹⁴ VAD	AECRTAQMT	CSAVAPAPGC	QLVLRQDFNQ	.SGLYCLNVS	LANGNGLAVA	STHVAVGGAS	Trans- PAASGTTLTV		
chicken_I	-----	-----	-----	-----	-----	-----	-----	-----		
chicken_S	-----	-----	-----	-----	-----	-----	-----	-----		
chicken_D	-----	-----	-----	-----	-----	-----	-----	-----		
human	-K-A-MEISS	PG-QPPAQRL	-QP-L-S-A-	---H-ILKG	G--T-----	--DT-S--V	--QLIMP-QE	AGLGQVP-I-		
mouse	-K-A-MDISS	PG-QPPAQRL	-QS-P-S-D-	---H-VLKG	G--T-----	--DA-S---	--QLV-P-QD	GGLGQAP-L-		
mouse_silv	-K-A-MDISS	PG-QPPAQRL	-QS-P-S-D-	---H-VLKG	G--T-----	--DA-S---	--QLV-P-QD	GGLGQAP-L-		
chicken_i	GLL...LIAA	ALGTAAYTYE	membrane region RVKYSPLLP ¹⁵ T	Cytoplasmic region APTAPRPHSW	LPPGATLRLI	LRQAFGGAPS	GESSPLLRAN	AV*		
chicken_I	-----	-----	-----	-----	-----	-----	-----	-----		
chicken_S	-----	-----	-----	-----	-----	-----	-----	-----		
chicken_D	-----	-----	-----	-----	-----	-----	-----	-----		
human	-I-...-VLM	-VVL-SLI-	-RLMKQDFSV	PQLPHSSSH	-RLPRIFCSC-I	--N---SGQ	Q--		
mouse	-I-...-VLV	-VVL-SLIH	HRLKKQGS.V	SQMPHGSTH	-RLPPVF-ARGL	--N---SGQ	Q--		
mouse_silv	-I-...-VLV	-VVL-SLIH	HRLKKQGS.V	SQMPHGSTH	-RLPPVF-ARGL	--N---SGQ	Q--		

TABLE 5
Polymorphic sequence motifs in *PMEL17* among chicken breeds with different alleles at the *Dominant white* locus

Breed	Allele	Exon 7 repeat ^a	Exon 10
Red jungle fowl, zoo population	<i>i</i>	A-A-A-C	A
Smyth Brown Line ^b	<i>i</i>	A-A-A-C	A
<i>Dun</i> ^c	<i>D</i>	A-A-A-C	C
White Leghorn, L13 and ADOL ^b	<i>I</i>	A-B-A	B
Polish Buff Laced ^b	<i>I</i>	A-B-A	B
Commercial broiler, line A ^b	<i>I</i>	A-B-A	B
<i>Smoky</i> ^b	<i>S</i>	A-B-A	B
Rhode Island Red ^b	<i>i</i>	A-B-A	A
Black Langshan ^b	<i>i</i>	A-B-A	A
Broiler line, White Plymouth Rock	<i>i</i>	A-A-C	A
Japanese Phoenix ^b	<i>i</i>	A-A-C	A
Light Brahma ^b	<i>i</i>	A-A-C	A
White Crested Black Polish ^b	<i>i</i>	A-A-C	A
New Hampshire Red ^b	<i>i</i>	A-A-C	A
Commercial broiler, line B ^b	<i>i</i>	A-A-C	A
Red jungle fowl, line UCD001 ^b	<i>i</i>	A-A	A
Black Australorp ^b	<i>i</i>	A-A	A
Commercial broiler, line C ^b	<i>i</i>	A-A	A
Fayoumi ^b	<i>i</i>	A	A

A, wild-type sequence; B, WAP insertion; C, LGTAA deletion.

^a See Table 4.

^b From the University of Arkansas, Fayetteville, AR.

the complete association between the presence of *Dominant white* and certain *PMEL17* sequence variants across breeds implies that the causative mutations must be in the very close vicinity of *PMEL17*. An inspection of the corresponding regions in human and mouse does not reveal any other candidate genes with an established function in the melanocyte. Furthermore, *Dun* arose independently of *Dominant white* but phenotypic similarities and segregation data indicate that the two mutations are allelic. The observation of a unique deletion in *Dun* occurring in the near vicinity of the WAP insertion associated with *Dominant white* is very intriguing. Finally, *Smoky* appeared in a White Leghorn line and is assumed to reflect a revertant allele at the *Dominant white* locus. The observation that this allele shares the *PMEL17* sequence with *Dominant white* but in addition carries a unique 12-bp deletion in the *PMEL17* exon 6 strongly supports our conclusion that *PMEL17* causes these plumage color variants.

The phenotypic expressions of *Dominant white* and *Dun* are also consistent with previous data on the role

of *PMEL17* in eumelanogenesis in the mouse. Our observation that all birds expressing any black pigment (eumelanin) carried at least one copy of the wild-type *PMEL17* allele implies that *Dominant white* blocks the production of eumelanin but allows production of red pheomelanin. This result is in perfect agreement with the general knowledge that *Dominant white* as well as *Dun* primarily inhibits the production of black pigment (ZIEHL and HOLLANDER 1987; SMYTH 1990). Interestingly, the *silver* mutation in the mouse also affects primarily the production of eumelanin (SILVERS 1979). These phenotypic effects in the chicken and mouse mutants are consistent with the crucial role of *PMEL17* (alias GP100) in the development of eumelanosomes but not of pheomelanosomes (KOBAYASHI *et al.* 1995).

Our data strongly suggest that the observed insertion/deletion polymorphisms in exons 6 and 10 are causative for the *Dominant white* and *Smoky* phenotypes. The causative nature of these mutations is supported by the complete associations observed and by the fact that we have sequenced all exons and all introns. Furthermore,

FIGURE 2.—Alignment of the *PMEL17* amino acid sequence associated with the wild-type (*I***i*) allele present in the red jungle fowl (RJF), and the *Dominant white* (*I***I*), *Dun* (*I***D*), and *Smoky* (*I***S*) alleles in chicken in comparison with human (S73003) and mouse (NM_021882) sequences including the mouse *silver* allele (AF119092). Sequence identities are indicated by dashes and insertion/deletion differences are indicated by dots. The signal sequence, the four copies of the 24-amino-acid repeat in chicken, the transmembrane, and the cytoplasmic region are indicated. The arrow indicates the proteolytic cleavage site that generates an aminoterminal M α and a carboxyterminal M β fragment. The insertion/deletion polymorphisms associated with *Dominant white*, *Dun*, and *Smoky* are boxed.

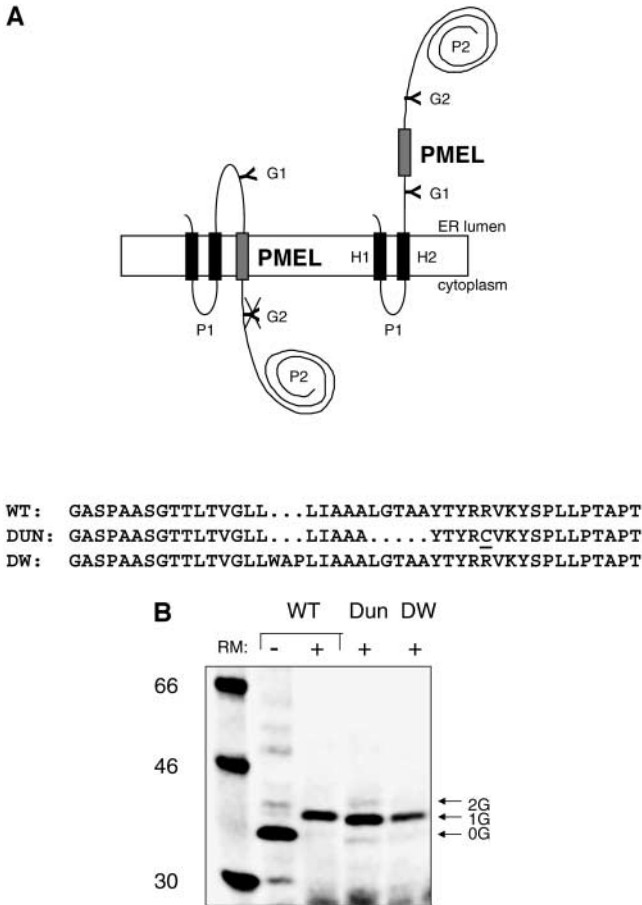


FIGURE 3.—Integration of PMEL17 transmembrane segments into microsomal membranes. (A) Wild-type Lep has two N-terminal TM segments (H1, H2) and a large luminal domain (P2). PMEL17-derived segments corresponding to the transmembrane region (PMEL) were inserted between residues 226 and 253 in the P2 domain. Glycosylation acceptor sites (G1, G2) were placed in positions 96–98 (Asn-Ser-Thr) and 258–260 (Asn-Ala-Thr), flanking the PMEL segment. For constructs with a PMEL segment that integrates into the membrane, only the G1 site is glycosylated (top left), whereas both the G1 and G2 sites are glycosylated for PMEL segments that do not integrate in the membrane (top right). (B) Membrane integration of PMEL17-derived transmembrane segments. Plasmids encoding the Lep-PMEL17 constructs were transcribed and translated *in vitro* in the absence (–RM) and presence (+RM) of dog pancreas rough microsomes. 0G, unglycosylated protein; 1G, singly glycosylated protein; 2G, doubly glycosylated protein. Molecular weight markers are shown in the first lane. WT, wild type; DW, Dominant white.

the expression analysis did not reveal any altered transcripts or any marked differences in expression levels in early embryos. *Dun* was found to be associated with three unique missense mutations at codons 35, 105, and 740 as well as with a unique deletion of five amino acids in the transmembrane region (Figure 2). The genetic data do not reveal which of these mutations is the causative one, but on the basis of the phenotypic similarity to *Dominant white* we postulate that the deletion in the transmembrane region is the most likely causative muta-

tion. We used the TMHMM2.0 program (KROGH *et al.* 2001; <http://www.cbs.dtu.dk/services/TMHMM/>), developed for the prediction of transmembrane helices (TMH) in proteins, to evaluate the consequences of the insertion/deletion polymorphisms associated with *Dominant white* and *Dun*. The program predicted the location of a TMH at the expected position in wild-type chicken PMEL17 with a posterior probability of 0.8. The insertion of WAP in the protein encoded by *Dominant white* reduced the probability to 0.6, most likely because the insertion of a proline residue may disturb the formation of a TMH. Furthermore, the *Dun*-associated deletion reduced the probability for the formation of a TMH even more, to a probability value <0.4. However, our *in vitro* experiments showed that the sequences encoded by both *Dominant white* and *Dun* could form TMHs. However, these insertion/deletion polymorphisms in and near the transmembrane segment may well affect PMEL17 function in other ways. It has been shown that PMEL17 is an integral membrane protein that is already present in stage I premelanosomes and that the cleavage and processing of this protein into an M α and M β subunit accompanies the restructuring of stage I melanosomes to stage II melanosomes with elongated fibrillar structures (KUSHIMOTO *et al.* 2001; BERSON *et al.* 2003). Even after PMEL17 has been cleaved to two subunits, the protein remains covalently tethered to its transmembrane domain (BERSON *et al.* 2001). Thus, it is conceivable that the insertion/deletion polymorphisms associated with *Dominant white* and *Dun* lead to a failure in the proper integration of PMEL17 in the melanosomal membrane, which disrupts the normal formation of eumelanosomes. In fact, previous ultrastructural studies have shown that the eumelanosomes in melanocytes from *I/i* heterozygotes are scarce and disorganized (BRUMBAUGH 1971).

Very few *PMEL17* mutations with phenotypic effects have been described so far in comparison with the large number of functionally important mutations described for other major coat-color loci like *Albino/TYR*, *Agouti*, and *Extension/MC1R*. In fact, only one mutation (*silver*) has been described so far in the mouse (<http://www.informatics.jax.org/>) and no human *PMEL17* mutation causing pigmentation disorders has yet been reported but it has been proposed as a likely candidate gene for some cases of human oculocutaneous albinism (<http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=155550>; December 2003). However, on the basis of the phenotypic effects in chicken and the established role for PMEL17 in the development of eumelanosomes but not of phaeomelanosomes, we postulate that this locus may be one of the loci causing red hair in humans. It is intriguing that *Dominant white* and *Dun* in chicken and *silver* in mouse are all insertion/deletions affecting the transmembrane or cytoplasmic region. No mutation with a phenotypic effect, except *Smoky*, has been found in the part of the protein forming the M α subunit.

Furthermore, no mutation causing a total loss of the PMEL17 protein has yet been found in any species despite the fact that such a mutation is expected to give an obvious pigmentation phenotype. This implies that such mutations may be lethal. PMEL17 may have a hitherto unknown crucial function outside the melanocytes. The absence of melanogenesis does not have a severe effect on survival as well documented by the viability of albino mutants in a variety of species. The suggestion that PMEL17 may have an important role in nonpigment cells gains some support by the fact that human EST sequences for PMEL17 have been obtained from a variety of tissues, although the majority of them are from melanoma cells (<http://www.ncbi.nlm.nih.gov/UniGene>). The rare occurrence of *PMEL17* mutations suggests that the chicken mutations described here should be useful for structural/functional studies of this protein.

An exciting topic for future studies will be to unravel how the four-amino-acid deletion associated with *Smoky* can partly rescue the defect caused by the WAP insertion in *Dominant white*. PMEL17 forms fibers after it has been cleaved by furin into an M α and M β fragment. This process appears to be essential for the normal development of eumelanosomes. A reasonable interpretation therefore is that the insertion of the WAP amino acid triplet disrupts the process but it is partially restored by the *Smoky* deletion. This would resemble the trans-suppression of transthyretin misfolding in composite heterozygotes that protects from the development of amyloid disease in humans (HAMMARSTROM *et al.* 2001).

The segregation analysis of *PMEL17* alleles and plumage color in our F₂ generation showed that *Dominant white* is required but not sufficient for the expression of the completely white phenotype. Homozygosity for *Dominant white* increased the incidence of white color but other loci contributed as well. At least three other major loci segregate in this cross: *Extended black/MC1R*, *Silver*, and *Barred* (KERJE *et al.* 2003b). We have previously shown that homozygosity for the *Extended black* (*MC1R**E92K) allele increases the chance to develop a purely white phenotype, which may appear counterintuitive. However, it makes sense in light of the action of the *Dominant white/PMEL17* mutation since the *Extended black* allele shifts melanin production toward eumelanogenesis, which in turn is severely inhibited by the effect of *Dominant white*. This is consistent with the fact that *MC1R* acts upstream of *PMEL17* in the melanogenesis. Another major locus affecting plumage color in this intercross is the sex-linked *Silver* locus (not homologous to *silver* in the mouse!) where the dominant *S* allele inherited from the White Leghorn inhibits the expression of red pheomelanin. The causative gene for *Silver* has not yet been identified.

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LITERATURE CITED

- BATESON, W., 1902 Experiments with poultry. Rep. Evol. Commun. Roy. Soc. 1: 87–124.
- BERSON, J. F., D. C. HARPER, D. TENZA, G. RAPOSO and M. S. MARKS, 2001 Pmel17 initiates premelanosome morphogenesis within multivesicular bodies. Mol. Biol. Cell 12: 3451–3464.
- BERSON, J. F., A. C. THEOS, D. C. HARPER, D. TENZA, G. RAPOSO *et al.*, 2003 Proprotein convertase cleavage liberates a fibrillogenic fragment of a resident glycoprotein to initiate melanosome biogenesis. J. Cell Biol. 161: 521–533.
- BRUMBAUGH, J. A., 1971 The ultrastructural effects of the I and S loci upon black-red melanin differentiation in the fowl. Dev. Biol. 24: 392–412.
- DU, J., A. MILLER, H. R. WIDLUND, M. A. HORSTMANN, S. RAMASWAMY *et al.*, 2003 MLANA/MART1 and SILV/PMEL17/GP100 are transcriptionally regulated by MITF in melanocytes and melanoma. Am. J. Pathol. 163: 333–343.
- DUNNINGTON, E. A., and P. B. SIEGEL, 1996 Long-term divergent selection for eight-week body weight in White Plymouth rock chickens. Poultry Sci. 75: 1168–1179.
- GREEN, P., K. FALLS and S. CROOK, 1990 Documentation for CRIMAP, Version 2.4. Washington University School of Medicine, St. Louis.
- HAMMARSTROM, P., F. SCHNEIDER and J. W. KELLY, 2001 Trans-suppression of misfolding in an amyloid disease. Science 293: 2459–2462.
- HESSA, T., M. MONNÉ and G. VON HEIJNE, 2003 Stop-transfer efficiency of marginally hydrophobic segments depends on the length of the C-terminal tail. EMBO Rep. 4: 178–183.
- HURST, C. C., 1905 Experiments with poultry. Rep. Evol. Commun. Roy. Soc. 2: 131–154.
- JOHANSSON, M., I. M. NILSSON and G. VON HEIJNE, 1993 Positively charged amino acids placed next to a signal sequence block protein translocation more efficiently in *Escherichia coli* than in mammalian microsomes. Mol. Gen. Genet. 239: 251–256.
- KERJE, S., Ö. CARLBORG, L. JACOBSSON, K. SCHÜTZ, C. HARTMANN *et al.*, 2003a The twofold difference in adult size between the red jungle fowl and White Leghorn chickens is largely explained by a limited number of QTLs. Anim. Genet. 34: 264–274.
- KERJE, S., J. LIND, K. SCHÜTZ, P. JENSEN and L. ANDERSSON, 2003b Melanocortin 1-receptor (*MC1R*) mutations are associated with plumage color in the chicken. Anim. Genet. 34: 241–248.
- KOBAYASHI, T., W. D. VIEIRA, B. POTTERF, C. SAKAI, G. IMOKAWA *et al.*, 1995 Modulation of melanogenic protein expression during the switch from eu- to pheomelanogenesis. J. Cell Sci. 108: 2301–2309.
- KROGH, A., B. LARSSON, G. VON HEIJNE and E. L. L. SONNHAMMER, 2001 Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J. Mol. Biol. 305: 567–580.
- KUSHIMOTO, T., V. BASRUR, J. VALENCIA, J. MATSUNAGA, W. D. VIEIRA *et al.*, 2001 A model for melanosome biogenesis based on the purification and analysis of early melanosomes. Proc. Natl. Acad. Sci. USA 98: 10698–10703.
- MARTINEZ-ESPARZA, M., C. JIMENEZ-CERVANTES, D. C. BENNETT, J. A. LOZANO, F. SOLANO *et al.*, 1999 The mouse *silver* locus encodes a single transcript truncated by the silver mutation. Mamm. Genome 10: 1168–1171.
- MOCHII, M., K. AGATA and G. EGUCHI, 1991 Complete sequence and expression of a cDNA encoding a chicken 115-kDa melanosomal matrix protein. Pigment Cell Res. 4: 41–47.
- QUEVEDO, W., C. R. D. FLEISCHMANN and J. DYCKMAN, 1981 Premature loss of melanocytes from hair follicles (*B^h*) of light and silver (*si*) mice, pp. 177–183 in *Pigment Cell: Phenotypic Expression in Pigment*, edited by M. SEIJI. Tokyo University Press, Tokyo.
- RUYTER-SPIRA, C. P., Z. L. GU, J. J. VAN DER POEL and M. A. M. GROENEN, 1996 Bulk segregant analysis using microsatellite: mapping the Dominant White locus in the chicken. Poultry Sci. 76: 386–391.
- SÄÄF, A., E. WALLIN and G. VON HEIJNE, 1998 Stop-transfer function of pseudo-random amino acid segments during translocation across prokaryotic and eukaryotic membranes. Eur. J. Biochem. 251: 821–829.
- SCHMID, M., I. NANDA, M. GUTTENBACH, C. STEINLEIN, H. HOEHN *et al.*, 2000 First report on chicken genes and chromosomes 2000. Cytogenet. Cell Genet. 90: 169–218.

- SILVERS, W. K., 1979 *The Coat Colors of Mice: A Model for Mammalian Gene Action and Interaction*. Springer-Verlag, New York.
- SMYTH, J. R., 1990 Genetics of plumage, skin and pigmentation in chickens, pp 109–167 in *Poultry Breeding and Genetics*, edited by R. D. CRAWFORD. Elsevier Science, New York.
- SOLANO, F., M. MARTINEZ-ESPERANZA, C. JIMENEZ-CERVANTES, S. P. HILL, J. A. LOZANO *et al.*, 2000 New insight on the structure of the mouse *silver* locus and on the function of the *silver* protein. *Pigment Cell Res.* **13**: 118–124.
- ZIEHL, M. A., and W. F. HOLLANDER, 1987 DUN, a new plumage-color mutant at the I-locus in the fowl (*Gallus gallus*). *Iowa State J. Res.* **62**: 337–342.

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