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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 melanins 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 by footnote *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 μl containing ~50 ng DNA, 1× PCR buffer (QIAGEN, Valencia, CA), 2.5 mM $MgCl_2$, 200 μM 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× 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 sequncing. 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 μl 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.l20; 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 $MgCl_2$ 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 μl 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 μl including ~20 ng cDNA, 1× PCR Buffer (QIAGEN), 2.5 mM $MgCl_2$, 200 μM 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 μM 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 $MgCl_2$, 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, *Spe*I- and *Kpn*I-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 μl SP6 express TnT reaction mix (Promega), 0.5 μg of plasmid, 7.5 μCi ^{35}S (Amersham Bioscience), and 1 μl (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 F_2 birds in our red jungle fowl × 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 F_2 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	*PMEL17*	0	107.2
I	*MYG1*	0.13	26.4
I	*MCW188*	0.09	32.8
I	*MCW317*	0.21	6.7
PMEL17	*MYG1*	0.12	75.9
PMEL17	*MCW188*	0.10	70.8
PMEL17	*MCW317*	0.28	8.3
MYG1	*MCW188*	0.14	41.8
MYG1	*MCW317*	0.14	24.7
MCW188	*MCW317*	0.24	7.9

θ, recombination fraction; *Z*, LOD score.

TABLE 2

Plumage color and *PMEL17* genotype distributions in the F_2 generation of a White Leghorn/red jungle fowl intercross

		PMEL17			
Phenotype class	*I* genotype[a]	*W/W*	*J/W*	*J/J*	Total
	Females				
White	*I/−*	91	120	1[d]	212
Cream[b]	*I/−*	5	35	1[d]	41
White with black spots	*I/−*	0	33	1[d]	34
Colored[c]	*i/i*	0	0	103	103
Total for females		96	188	106	390
	Males				
White	*I/−*	32	46	0	78
Cream[b]	*I/−*	12	13	0	25
White with red/brown	*I/−*	46	155	0	201
White with black spots	*I/−*	0	2	0	2
Colored[c]	*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.

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 *MC1R* 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 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 F_2 birds were hemizygous for these dominant alleles inherited from the White Leghorn F_0 females. In males, 50% of the F_2 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*)	Black Langshan (*i/i*)	Broiler line (*i/i*)	White Leghorn (*I/I*)	*Smoky* (*S/S*)	*Dun* (*D/D*)
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	
	1626	C		T			
Exon 6	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	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*)	Black Langshan (*i/i*)	Broiler line (*i/i*)	White Leghorn (*I/I*)	*Smoky* (*S/S*)	*Dun* (*D/D*)
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.

[a] This 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

	Composition of repeats
Repeat type A	TAGATDGDAVGPTAAATAESIADP
Repeat type B	----------....----------
Repeat type C	IV----------------------

	Amino acid residue										
Breed	34	35	105	232	280-4	399	Repeat	586	723-5	731-5	740
Red jungle fowl (*i/i*)	S	A	G	A	PTVT	N	A-A-A-C	E		LGTAA	R
Black Langshan (*i/i*)	P	–	–	–	–	D	A-B-A	–	–	–	–
Broiler line (*i/i*)	–	–	–	V	–	D	A-A-C	K	–	–	–
White Leghorn (*I/I*)	–	–	–	–	–	D	A-B-A	–	WAP	–	–
Smoky (*S/S*)	–	–	–	–	del	D	A-B-A	–	WAP	–	–
Dun (*D/D*)	–	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 F_4 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 F_2 progeny in our red jungle fowl/White Leghorn intercross were homozygous for the *PMEL17* allele inherited from the red jungle fowl. All F_2 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 F_2 birds and

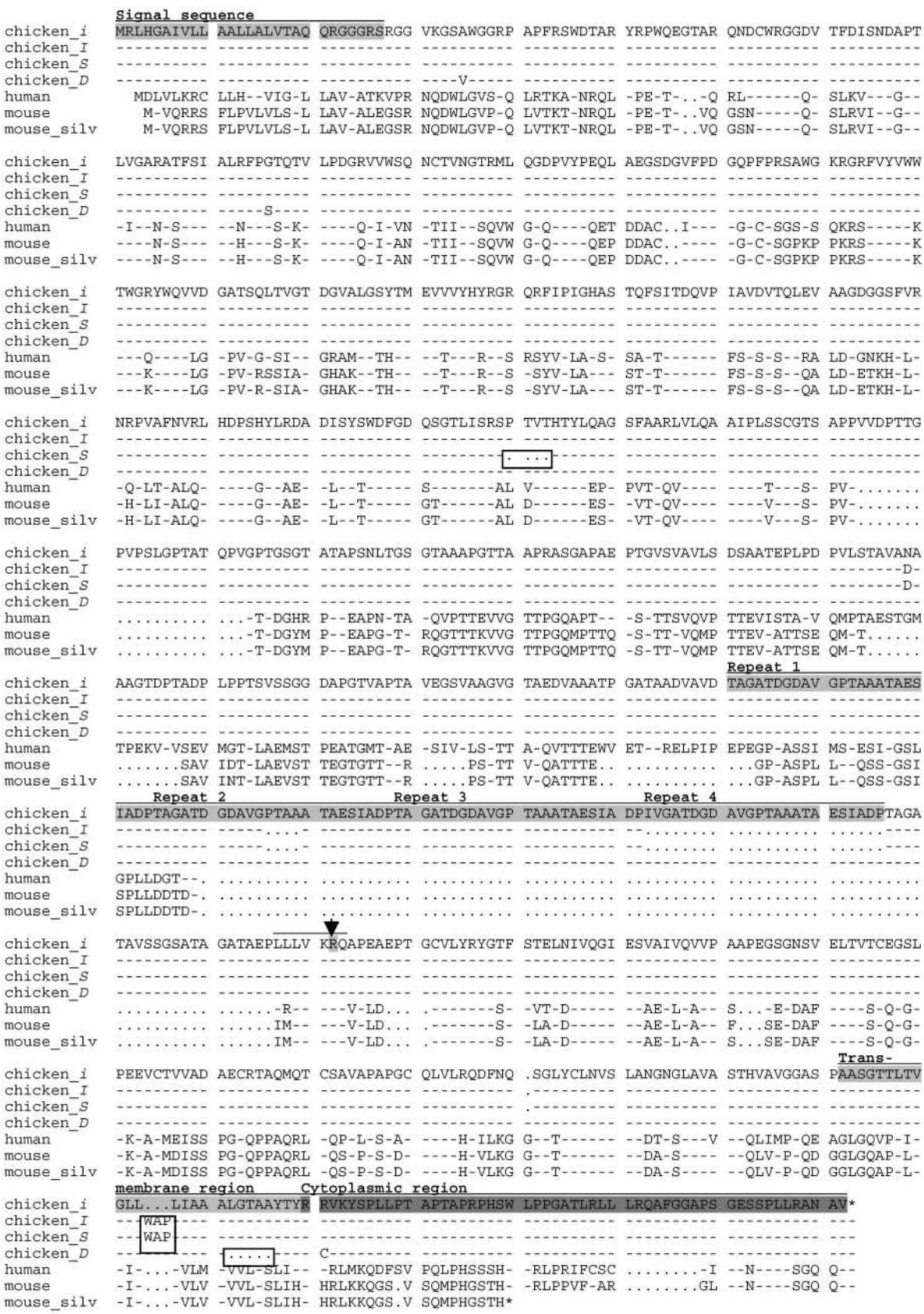
Signal sequence
chicken_i MRLHGAIVLL AALLALVTAQ QRGGGRSRGG VKGSAWGGRP APFRSWDTAR YRPWQEGTAR QNDCWRGGDV TFDISNDAPT
chicken_I ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
chicken_S ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
chicken_D ---------- ---------- ---------- ----V----- ---------- ---------- ---------- ----------
human MDLVLKRC LLH--VIG-L LAV-ATKVPR NQDWLGVS-Q LRTKA-NRQL -PE-T-..-Q RL------Q- SLKV---G--
mouse M-VQRRS FLPVLVLS-L LAV-ALEGSR NQDWLGVP-Q LVTKT-NRQL -PE-T-..VQ GSN-----Q- SLRVI--G--
mouse_silv M-VQRRS FLPVLVLS-L LAV-ALEGSR NQDWLGVP-Q LVTKT-NRQL -PE-T-..VQ GSN-----Q- SLRVI--G--
chicken_i LVGARATFSI ALRFPGTQTV LPDGRVVWSQ NCTVNGTRML QGDPVYPEQL AEGSDGVFPD GQPFPRSAWG KRGRFVYVWW
chicken_I ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
chicken_S ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
chicken_D ---------- -----S---- ---------- ---------- ---------- ---------- ---------- ----------
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 TWGRYWQVVD GATSQLTVGT 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 HDPSHYLRDA 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 GTAAAPGTTA APRASGAPAE PTGVSVAVLS DSAATEPLPD PVLSTAVANA
chicken_I ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------D-
chicken_S ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------D-
chicken_D ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
human ....-T-DGHR P--EAPN-TA -QVPTTEVVG TTPGQAPT-- -S-TTSVQVP TTEVISTA-V QMPTAESTGM
mouse ....-T-DGYM P--EAPG-T- RQGTTTKVVG TTPGQMPTTQ -S-TT-VQMP TTEV-ATTSE QM-T......
mouse_silv ....-T-DGYM P--EAPG-T- RQGTTTKVVG TTPGQMPTTQ -S-TT-VQMP TTEV-ATTSE QM-T......
Repeat 1
chicken_i AAGTDPTADP LPPTSVSSGG DAPGTVAPTA VEGSVAAGVG TAEDVAAATP GATAADVAVD TAGATDGDAV 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
mouse ....SAV IDT-LAEVST TEGTGTT--R .....PS-TT V-QATTTE.. ....GP-ASPL L--QSS-GSI
mouse_silv ....SAV INT-LAEVST TEGTGTT--R .....PS-TT V-QATTTE.. ....GP-ASPL L--QSS-GSI
Repeat 2 Repeat 3 Repeat 4
chicken_i IADPTAGATD GDAVGPTAAA TAESIADPTA GATDGDAVGP TAAATAESIA DPIVGATDGD AVGPTAAATA ESIADPTAGA
chicken_I ---------- -----....- ---------- ---------- ---------- ---------- --........ ....
chicken_S ---------- -----....- ---------- ---------- ---------- ---------- --........ ....
chicken_D ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
human GPLLDGT--. ....
mouse SPLLDDTD-. ....
mouse_silv SPLLDDTD-. ....
chicken_i TAVSSGSATA GATAEPLLLV KRQAPEAEPT 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-
mouse ....IM-- ---V-LD... .-------S- -LA-D----- --AE-L-A-- F...SE-DAF ----S-Q-G-
mouse_silv ....IM-- ---V-LD... .-------S- -LA-D----- --AE-L-A-- S...SE-DAF ----S-Q-G-
Trans-
chicken_i PEEVCTVVAD AECRTAQMQT CSAVAPAPGC QLVLRQDFNQ .SGLYCLNVS LANGNGLAVA STHVAVGGAS 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-
membrane region Cytoplasmic region
chicken_i GLL...LIAA ALGTAAYTYR RVKYSPLLPT APTAPRPHSW LPPGATLRLL LRQAFGGAPS GESSPLLRAN AV*
chicken_I ---WAP---- ---------- ---------- ---------- ---------- ---------- ---------- ---
chicken_S ---WAP---- ---------- ---------- ---------- ---------- ---------- ---------- ---
chicken_D ---------- ----.----- C--------- ---------- ---------- ---------- ---------- ---
human -I-...-VLM -VVL-SLI-- -RLMKQDFSV PQLPHSSSH- -RLPRIFCSC ....-I --N----SGQ Q--
mouse -I-...-VLV -VVL-SLIH- HRLKKQGS.V SQMPHGSTH- -RLPPVF-AR ....GL --N----SGQ Q--
mouse_silv -I-...-VLV -VVL-SLIH- HRLKKQGS.V SQMPHGSTH*

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*	A-A-A-C	A
Smyth Brown Line[b]	*i*	A-A-A-C	A
Dun[c]	*D*	A-A-A-C	C
White Leghorn, L13 and ADOL[b]	*I*	A-B-A	B
Polish Buff Laced[b]	*I*	A-B-A	B
Commercial broiler, line A[b]	*I*	A-B-A	B
Smoky[b]	*S*	A-B-A	B
Rhode Island Red[b]	*i*	A-B-A	A
Black Langshan[b]	*i*	A-B-A	A
Broiler line, White Plymouth Rock	*i*	A-A-C	A
Japanese Phoenix[b]	*i*	A-A-C	A
Light Brahma[b]	*i*	A-A-C	A
White Crested Black Polish[b]	*i*	A-A-C	A
New Hampshire Red[b]	*i*	A-A-C	A
Commercial broiler, line B[b]	*i*	A-A-C	A
Red jungle fowl, line UCD001[b]	*i*	A-A	A
Black Australorp[b]	*i*	A-A	A
Commercial broiler, line C[b]	*i*	A-A	A
Fayoumi[b]	*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 Mα and a carboxyterminal Mβ fragment. The insertion/deletion polymorphisms associated with *Dominant white*, *Dun*, and *Smoky* are boxed.

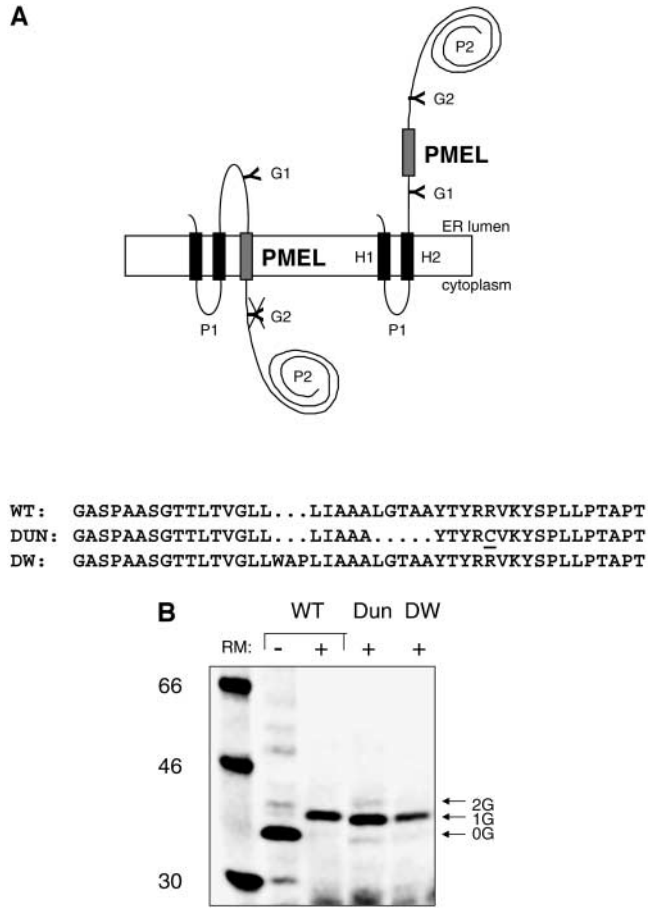

FIGURE 3.—Integration of PMEL17 transmembrane segments into microsomal membranes. (A) Wild-type Lep has two N-terminal TM segments (H1, H2) and a large luminal domain (P2). PMEL17-derived segments corresponding to the transmembrane region (PMEL) were inserted between residues 226 and 253 in the P2 domain. 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 mutation. 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 Mα and Mβ 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 Mα 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 Mα and Mβ 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 transsuppression 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 F_2 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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