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Mutations in *SLC45A2* Cause Plumage Color Variation in Chicken and Japanese Quail

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ABSTRACT

*S*S* (*Silver*), *S*N* (*wild type/gold*), and *S*AL* (*sex-linked imperfect albinism*) form a series of alleles at the *S* (*Silver*) locus on chicken (*Gallus gallus*) chromosome Z. Similarly, *sex-linked imperfect albinism* (*AL*A*) is the bottom recessive allele at the orthologous *AL* locus in Japanese quail (*Coturnix japonica*). The solute carrier family 45, member 2, protein (SLC45A2), previously denoted membrane-associated transporter protein (MATP), has an important role in vesicle sorting in the melanocytes. Here we report five *SLC45A2* mutations. The 106delT mutation in the chicken *S*AL* allele results in a frameshift and a premature stop codon and the corresponding mRNA appears to be degraded by nonsense-mediated mRNA decay. A splice-site mutation in the Japanese quail *AL*A* allele causes in-frame skipping of exon 4. Two independent missense mutations (Tyr277Cys and Leu347Met) were associated with the *Silver* allele in chicken. The functional significance of the former mutation, associated only with *Silver* in White Leghorn, is unclear. Ala72Asp was associated with the *cinnamon* allele (*AL*C*) in the Japanese quail. The most interesting feature concerning the *SLC45A2* variants documented in this study is the specific inhibition of expression of red pheomelanin in *Silver* chickens. This phenotypic effect cannot be explained on the basis of the current, incomplete, understanding of SLC45A2 function. It is an enigma why recessive null mutations at this locus cause an almost complete absence of both eumelanin and pheomelanin whereas some missense mutations are dominant and cause a specific inhibition of pheomelanin production.

PIGMENTATION in birds and mammals is based on the synthesis of two different types of melanin, brown/black eumelanin and yellow/red pheomelanin. Tyrosinase is the rate-limiting enzyme of melanin biosynthesis, which takes place in melanosomes within melanocytes. Tyrosinase and the tyrosinase-related proteins Tyrp1 and Tyrp2 (Dct) are involved in the production of eumelanin. Only the presence of cysteine and some tyrosinase activity appear to be required for the production of pheomelanin. When tyrosinase is expressed at low levels, pheomelanin is produced by the addition of cysteine to dopaquinone (Kobayashi *et al.* 1995; Wakamatsu and Ito 2002; Kushimoto *et al.* 2003). Simplified, high tyrosinase activity is associated with synthesis of eumelanin whereas low activity results in the production of pheomelanin. The spherical pheomelanin premelanosomes are less organized than the rod-shaped eumelanin premelanosomes and contain less melanin (Brumbaugh 1968).

In birds, females are the heterogametic sex (ZW) and males the homogametic sex (ZZ). The sex-linked *Silver* locus controlling *Silver* (*S*S*) and *wild type/gold* (*S*N*) plumage color (Figure 1) in chicken (*Gallus gallus*) was described in 1912 by Sturtevant, who found a sex-linked factor (*Silver*) that inhibits red color pigmentation (Sturtevant 1912). *Silver* is incompletely dominant to *wild type* and its phenotypic expression is highly influenced by modifying genes. It can thus be difficult to identify *Silver* in some genetic backgrounds (Smyth 1990). *Sex-linked imperfect albinism* (*S*AL*) is the bottom recessive allele at this locus (Werret *et al.* 1959; Cole and Jeffers 1963) (Figure 1). *S*AL* birds have white plumage with a ghost pattern that depends on genetic background. The eyes are pink at hatching but darken with age except for the red pupils (Mueller and Hutt 1941; Hutt and Mueller 1943; Werret *et al.* 1959; Silversides and Crawford 1990).

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. DQ900684, DQ900688–DQ900700 (chicken), and EF031011–EF031013 (Japanese quail).

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Figure 1.—Chickens expressing the *wild type* (*S*N*), *Silver* (*S*S*), and *sex-linked imperfect albinism* (*S*AL*) phenotypes.

The recessive allele for sex-linked albinism in the Japanese quail (*Coturnix japonica*) was designated *AL*A*, while the wild-type allele was denoted *AL*N* (Lauber 1964; Minvielle *et al.* 2000) (Figure 2). By intergeneric crossing of male chickens (*G. gallus*) homozygous for *S*S*, *S*N*, or *S*AL* to female Japanese quail (*C. japonica*) hemizygous for *AL*N* or *AL*A*, it was found that *S* in chicken and *AL* in quail are orthologous. All five intergeneric hybrids from the crossing of albinos from the two Phasianidae species were albino (Silversides and Mérat 1991). *Cinnamon* (*AL*C*) is another allele at the *AL* locus in the Japanese quail (Figure 2). Both the *AL*C* and the *AL*A* mutations are caused by recessive alleles, and *AL*C* is dominant over *AL*A* (Truax and Johnson 1979; Cheng and Kimura 1990; Minvielle *et al.* 2000). Thus, the hierarchy of dominance for these alleles is *AL*N* > *AL*C* > *AL*A*. The plumage of wild-type Japanese quail is brown in variable shades (Figure 2). The quail albino chicks have bright pink eyes and yellow to white color. The adult birds have white plumage with buff ghost barring (Lauber 1964; Cheng and Kimura 1990). *Cinnamon* is phenotypically identical to the *dark-eyed dilute* (*AL*D*) allele (Cheng and Kimura 1990). The eyes of the *AL*D* chicks are red and have subnormal melanin pigmentation but darken with age. The *AL*D* mutation results in dilution of the brown pigment of the feathers but the plumage pattern is not affected (Cheng and Kimura 1990).

The gene encoding solute carrier family 45, member 2, protein (*SLC45A2*) is associated with pigmentation variation in several vertebrates; *SLC45A2* was previously known as membrane-associated transporter protein (*MATP*). *SLC45A2* mutations have been found in medaka (Fukamachi *et al.* 2001), humans (Newton *et al.* 2001), mouse (Newton *et al.* 2001; Du and Fisher 2002), and horse (Mariat *et al.* 2003). Oculocutaneous albinism type IV (OCA4) in humans is caused by mutations in *SLC45A2* (Newton *et al.* 2001). SLC45A2 has 12 predicted transmembrane regions (Fukamachi *et al.* 2001) but the function of SLC45A2 is not fully understood. Mutations in *SLC45A2* have been shown to disrupt tyrosinase processing and trafficking at the post-Golgi level (Kushimoto *et al.* 2003; Hearing 2005). SLC45A2 was first identified as an antigen in melanoma (AIM), AIM1 (Harada *et al.* 2001).

The sex-linked *Silver* locus in chicken is known to be located on the upper half of chicken chromosome Z, 2.4 cM from the slow-feathering (*K*) locus (Bitgood 1999), which is tightly linked to the chicken endogenous virus *ev*21 (Bacon *et al.* 1988). *SLC45A2* is located in the vicinity of this viral insertion (within a 300-kb distance on chicken chromosome Z, http://www.genome.ucsc.edu). Here we show that mutations in *SLC45A2* cause *imperfect albinism* both in chicken (*S*AL*) and in Japanese quail (*AL*A*) as well as the *Silver* (*S*S*) and *cinnamon* (*AL*C*) phenotypes in the two species.

MATERIALS AND METHODS

Animals: A number of chicken pedigrees and breeds have been used in this study. A three-generation pedigree from an intercross between one red jungle fowl (RJF) male and three

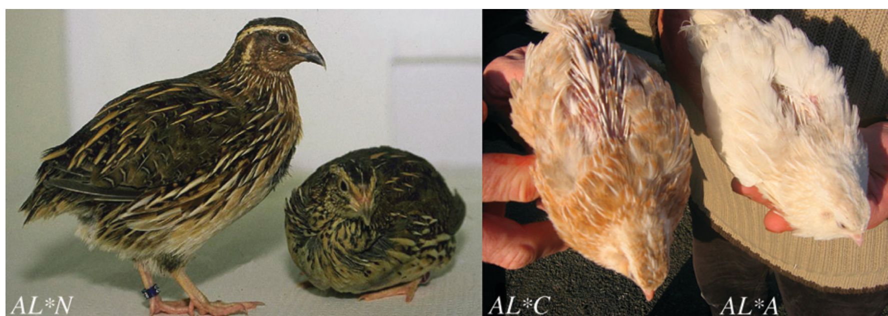

Figure 2.—Japanese quails expressing the *wild type* (*AL*N*), *cinnamon* (*AL*C*), and *sex-linked imperfect albinism* (*AL*A*) phenotypes.

White Leghorn (WL) females has been generated for gene mapping (Schutz *et al.* 2002). From the F_1 generation four males and 37 females were selected to generate an F_2 generation, showing a wide diversity of plumage color (Kerje *et al.* 2003). Two other family materials were also used. The first segregated for *Silver* (White Buttercup, *S*S vs.* Brown Buttercup, *S*N*) and is an experimental cross, developed for the identification of plumage color genes. The origins of the cross are a broiler line and a brown layer line. The second segregated for *Silver (S*S)* and *imperfect albinism (S*AL*) and is a synthetic population (Mérat *et al.* 1986) currently raised at the INRA Génétique Factorielle Avicole (GFA) experimental unit in Nouzilly. The birds carrying *S*AL* were received from R. G. Somes in Connecticut in 1979. In the 1980s these birds were crossed with a synthetic French line carrying *Silver.* Rhode Island Red (*S*N*) has also been crossed into this population. A total of 50 animals exhibiting different genotypes at the *S* locus were sampled from this gene pool. DNA samples from different chicken breeds collected by the AvianDiv project (Hillel *et al.* 2003), red jungle fowl DNA from four different Scandinavian zoo populations (Håkansson and Jensen 2005), and White Leghorn DNA from the hypothyroid obese strain (OS) (Cole 1966) were also used.

Japanese quail DNA samples from wild type (*AL*N*), *cinnamon* (*AL*C*), and *imperfect albinism* (*AL*A*) raised at the INRA GFA experimental unit in Nouzilly were used for sequencing. Skin tissue samples used for RNA extractions were from *AL*N* and *AL*A* quails and heterozygous (*S*S/S*AL* or *S*N/S*AL*) male chicks.

Sequencing of genomic DNA: Primers for amplification and sequencing of all *SLC45A2* exons for both chicken and quail were designed using *in silico* predicted intron–exon boundaries and DNA sequences from the February 2004 chicken genome assembly (http://www.genome.ucsc.edu). In chicken, the gene was amplified in six parts, using the primer pairs ex1Fpcr/ex1Rpcr, ex2Fpcr/ex2Rpcr, ex3Fpcr/ex3Rpcr, ex4&5Fpcr/ex4&5Rpcr, ex6Fpcr/ex6Rpcr, and ex7Fpcr/ex7Rpcr; both the PCR primers and internal sequencing primers were used for direct sequencing (supplemental Table 1 at http://www.genetics.org/supplemental/). Up- and downstream regions of chicken *SLC45A2* were sequenced using the primer pairs up10kbF/up10kbR, up20kbF/up20kbR, dwn9kbF/dwn9kbR, dwn20kbF/dwn20kbR, dwn30kbF/dwn30kbR, and dwn80kbF/dwn80kbR.

Due to the difficulties in amplifying quail DNA with chicken primers some additional primers were designed to amplify the quail exons. In quail, the gene was finally amplified in six parts, using the primer pairs ex1Fpcr/ex1Rpcr, ex2Fpcr/ex2Rseq, ex3Fseq/ex3Rpcr3, ex4&5Fpcr/ex4&5Rpcr, ex6Fpcr3/ex6Rpcr3, ex7Fpcr3/ex7Rpcr3, and ex7Fpcr5/ex7Rpcr5 (supplemental Table 1 at http://www.genetics.org/supplemental/).

All PCR reactions were carried out in a total volume of 10–20 μl and contained ~50 ng genomic DNA, 1× PCR buffer II (Applied Biosystems, Foster City, CA), 2.5 mm $MgCl_2$, 200 μm dNTPs, 0.75–1 unit AmpliTaq Gold DNA polymerase (Applied Biosystems), and 20 pmol of each primer. The PCRs were performed in an Applied Biosystems 2720 thermal cycler and started with 5 min at 94°, followed by a touchdown PCR program starting with denaturation at 94° for 30 sec, annealing for 30 sec, and elongation at 72° for 1 min/kb of PCR product. The annealing temperature started at 65° and was then decreased by 1°/cycle for 14 cycles, an additional 30 cycles was run on 51° constant annealing temperature, and the last cycle ended with 72° for 10 min.

PCR fragments were gel purified using the E.Z.N.A gel extraction kit (Omega Bio-tek, Doraville, GA) and sequenced directly using the DYEnamic ET dye terminator kit (MegaBACE) and the MegaBACE 1000 instrument (GE Healthcare Bio-Sciences, Uppsala, Sweden). Sequences were analyzed with Sequence Analysis software (GE Healthcare Bio-Sciences) and Sequencher 3.1.1 software (Gene Codes, Ann Arbor, MI).

cDNA sequencing plus 5′ and 3′ RACE: The First-Strand cDNA Synthesis kit (GE Healthcare Bio-Sciences) was used for cDNA synthesis from 14-day-old whole-embryo chicken total RNA (Kerje *et al.* 2004). PCR amplifications were performed using the cDNAexon1F and cDNAexon7R primers (supplemental Table 1 at http://www.genetics.org/supplemental/) and the reactions were carried out as described above but with minor changes: 130 ng of cDNA per reaction and 35 cycles on 51° constant annealing temperature. The PCR fragment was cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced using the T7 and M13 universal primers. Primers for 5′ and 3′ RACE were designed on the basis of the obtained sequences.

5′ and 3′ RACE were performed on RACE-ready first-strand cDNA from broiler brain according to the Gene Racer kit protocol (Invitrogen). The *SLC45A2*-specific primers used in this experiment, together with the primers provided by the kit, were chRACE5′rev1, chRACE3′fwd2, and the nested primer chRACE3′fwd1 (supplemental Table 1 at http://www.genetics.org/supplemental/). The RACE-ready first-strand cDNA was diluted 1:9 and 1 μl was used for PCR amplification in a total volume of 20 μl with 1× PCR buffer II (Applied Biosystems), 2 mm $MgCl_2$, 200 μm dNTPs, 1 unit AmpliTaq Gold DNA polymerase (Applied Biosystems), and 20 pmol of each primer. The PCR started with 5 min at 94°, followed by a touchdown PCR program starting with 94° for 30 sec, annealing for 30 sec, and elongation at 72° for 1 min. The annealing temperature started at 72° and was then decreased 1°/cycle for 9 cycles, an additional 40 cycles were run on 66° constant annealing temperature, and the last cycle ended with 72° for 10 min. The PCR fragments were cloned using the TOPO TA cloning kit (Invitrogen) and sequenced using the T7 and M13 universal primers.

Genotyping and linkage mapping: Pyrosequencing with Pyro Gold chemistry was used to analyze chicken coding SNPs in exon 3 and exon 4 (Biotage, Uppsala, Sweden). A 113-bp fragment containing the SNP at position 902 A → G in exon 3 was amplified with the PYROex3Fseq and PYROex3Rbio primers and a 104-bp fragment containing the SNP at position 1111 C → A in exon 4 was amplified with PYROex4Fbio and PYROex4Rseq primers (supplemental Table 1 at http://www.genetics.org/supplemental/). PCR reactions were carried out as described above with minor changes. For the PYROex3Fseq/PYROex3Rbio the PCR started with 5 min at 94°, followed by 45 cycles of 94° for 30 sec, 51° for 30 sec, and 72° for 15 sec, and the last cycle ended with 72° for 10 min. For the PYROex4Fbio/PYROex4Rseq the regular touchdown program was used with 40 cycles at 51° constant annealing temperature. The PYROex3Fseq and PYROex4Rseq were also used as sequencing primers in their respective tests and were designed to anneal just prior the SNP of interest. *SLC45A2* was mapped in relation to other markers genotyped in the RJF × WL pedigree using the TWOPOINT function in CRIMAP (Green *et al.* 1990). The BUILD and FLIPS functions were used to test the order of markers.

Confirmation of nonsense-mediated mRNA decay in *imperfect albino* chickens: Total RNA was isolated from skin of chicks heterozygous for the *S*AL* deletion using the RNeasy fibrous tissue mini kit (QIAGEN, Valencia, CA). The tissues had been stored in RNAlater (Ambion, Austin, TX) at −80° prior to extraction. The RNA concentration and purity were checked using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The RNA was treated with DNase according to the instructions for the DNA-*free* kit (Ambion) before cDNA synthesis using the First-Strand

cDNA Synthesis kit (GE Healthcare Bio-Sciences). The chSALtestF/chSALtestR2 primers (supplemental Table 1 at http://www.genetics.org/supplemental/) were used to amplify and sequence the cDNA from chicks heterozygous for *S*AL* to test if the *106delT* allele results in a downregulation of the mRNA due to nonsense-mediated mRNA decay (NMD). The reactions were carried out as described above with minor changes: 1 μl of the cDNA in a 20-μl reaction and 40 cycles at 51° constant annealing temperature. From the same individuals genomic DNA was extracted using standard methods and the ex1Fpcr/ex1Rpcr primers were used for amplification and sequencing as described above.

Expression analysis of the mutation causing *imperfect albinism* in Japanese quail: Total RNA was isolated and cDNA synthesized from quail skin as described above for chicken skin. The test was designed to check if the G → T mutation at the splice acceptor site in the *imperfect albinism* allele at the intron3/exon 4 border results in an in-frame exon skipping. The AlQex3cDNAfwd/AlQex5cDNArev primers (supplemental Table 1 at http://www.genetics.org/supplemental/) were used for amplification of quail cDNA. The reactions were carried out as described above for the NMD test.

RESULTS

Assignment of *SLC45A2* to the chicken linkage map: The avian leucosis virus sequence (X54094) associated with the slow-feathering (*K*) locus was used as bait to search the May 2006 chicken genome assembly (http://www.genome.ucsc.edu) to find the chromosomal region harboring the *Silver* locus. A hit was found on chromosome Z around position 10.0 Mb. *SLC45A2* is located ∼300 kb apart from the retroviral insertion at position ∼9.7 Mb, consistent with a map distance of a few centimorgans between *K* and *Silver* (Bacon *et al.* 1988). *SLC45A2* was therefore identified as an obvious positional candidate gene for *Silver.* A 902 A → G SNP was found by direct sequencing of exon 3 and used to map *SLC45A2* in relation to other markers genotyped in our RJF × WL intercross. Multipoint analysis revealed the following map order and map distances: *ADL0022*–(12.4 cM)–*SLC45A2*–(16.1 cM)–*MCW0053*. The order of loci was supported by a LOD score of 29.4 compared to the second most probable order.

Sequence analysis of chicken *SLC45A2*: *Ab initio* predictions of transcripts from multiexon genes have been shown to be very error prone. GENSCAN, one of the best programs, has a sensitivity and specificity of ∼90% for detecting exons (Burge and Karlin 1997). Therefore, we decided to experimentally verify the exon composition of the full-length *SLC45A2* transcript. The cDNA sequence obtained from a 14-day-old whole chicken embryo and broiler brain (deposited in GenBank with accession no. DQ900684) differed to a large extent from the one predicted by GENSCAN regarding exon/intron organization. A considerable part of exon 3 was missing and the last part of the sequence was erroneously predicted with an extra exon instead of the stop codon in exon 7. However, there is a predicted transcript XP429218 (http://www.ncbi.nlm.nih.gov/entrez) that correctly predicts exons 1–7 of *SLC45A2* but also adds an exon 8 that we could not verify experimentally.

All seven exons, including the splice sites and adjacent intronic parts, of *SLC45A2* were sequenced from genomic DNA samples representing different *Silver* alleles to evaluate *SLC45A2* as a candidate gene for this locus. The analysis involved four red jungle fowl, eight *Silver* birds, one *imperfect albino*, and a domestic fowl assumed to carry the *S*N* allele. The *S*S* allele was associated with a C → A transition in exon 4 (Leu347-Met) affecting transmembrane region 7 (Figures 3 and 4). This mutation was associated with *Silver* in all chicken breeds carrying this allele (the *Silver*, Fayoumi, Yurlov Crower, Rhode White, Friesian Fowl, and White Buttercup populations), except White Leghorn. An A → G transition in exon 3 (Tyr277Cys) affecting a loop region was found in the White Leghorn lines also assumed to carry *Silver* (Figures 3 and 4).

A 1-bp deletion (106delT) was revealed in *S*AL*, resulting in a frameshift at codon 36 and thereby a stop codon in exon 1 (Figure 3). Since a *Bgl*I site was created by the 106del mutation, a PCR–RFLP analysis was set up using *Bgl*I digestion. The test revealed a complete concordance between the presence of the 106del mutation and the *imperfect albino* genotype among 44 chickens, 31 albino (28 hemizygous females, 3 homozygous males) and 13 heterozygous carriers. Furthermore, the 106del mutation was not found in 6 nonalbino females, showing either the gold or the silver phenotype. A comparison of genomic DNA and cDNA from *S*AL* heterozygotes strongly suggested that NMD leads to degradation of the *S*AL* transcript (Figure 5). Direct sequencing of genomic DNA confirmed that the two tested birds were heterozygous for the *S*AL* deletion. Sequencing of cDNA from the same animals revealed only transcripts from the wild-type allele.

By additional sequencing of the up- and downstream regions of *SLC45A2* across populations with different genotypes we could document that all chicken breeds carrying the L347M mutation had a minimum shared haplotype <35 kb, including the entire *SLC45A2* coding sequence (Figure 6). The minimum shared haplotype among three different White Leghorn lines was even larger and extended for the entire region (>100 kb) covered in this study (Figure 6). It is worth noting that one sequenced bird (M2021) from the Nutreco experimental cross and the sequenced *imperfect albino* (*S*AL*) shared the same haplotype as White Leghorns for the entire *SLC45A2* gene but not for the downstream region (Figure 6).

Sequence analysis of Japanese quail *SLC45A2*: *SLC45A2* in quail was sequenced using genomic DNA and chicken-specific primers. A mutation was found in the *imperfect albino* (*AL*A*) birds, causing a G → T transversion at the splice acceptor site just preceding exon 4. Sequencing of cDNA prepared from skin samples from these birds confirmed that exon 4 is not

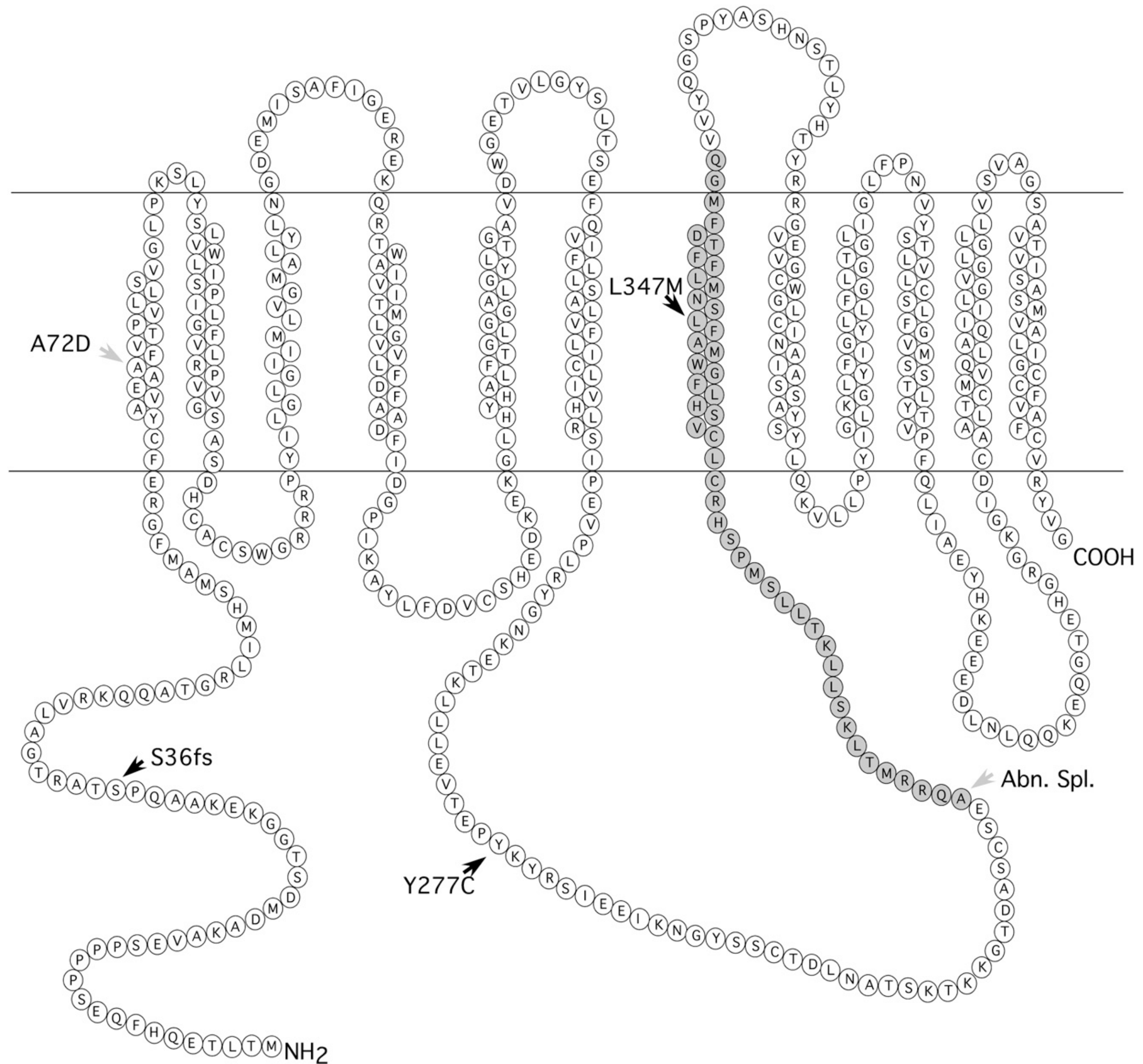

Figure 3.—Membrane topology prediction of the SLC45A2 protein using TMHMM (v. 2.0; http://www.cbs.dtu.dk/services/TMHMM-2.0/). The locations of the frameshift mutation (S36fs) associated with *imperfect albino* (*S*AL*) and the two missense mutations Y277C and L347M associated with *Silver* (*S*S*) in chicken are indicated by solid arrowheads. The A72D mutation associated with *cinnamon* (*AL*C*) in Japanese quail is marked with a shaded arrowhead. The missing amino acids in the SLC45A2 protein encoded by *sex-linked imperfect albinism* (*AL*A*) in Japanese quail are shaded. Abn.Spl., aberrant splicing.

present in the transcript (Figure 3). Another mutation was found in exon 1 of the *cinnamon* (*AL*C*) birds, causing a transition from C → A at nt 287 (Ala72Asp) (Figures 3 and 4).

A sequence comparison between the wild-type quail *vs.* the public red jungle fowl chicken genome sequence revealed a sequence identity in exons of 96.3%.

Association analysis across chicken breeds: A number of chicken breeds with or without the *Silver* phenotype and including four captive red jungle fowl populations were screened for the two missense mutations Y277C and L347M (Table 1). The L347M mutation showed an almost complete association with *Silver* across all populations presumed to carry this allele except the White Leghorns and it was not found in birds with the wild-type allele at this locus. We are convinced that the few discrepancies concerning the L347M mutation are due to phenotyping errors. Two White Buttercup (*S*S/S*N* or *S*S*/W) and five Brown Buttercup (*S*N/S*N* or *S*N*/W) birds showed discrepant *SLC45A2* genotypes (Table 1). These are most likely phenotyping errors since the scoring was done using 1-day-old chicks. At that age it can be difficult to unequivocally distinguish the phenotypes.

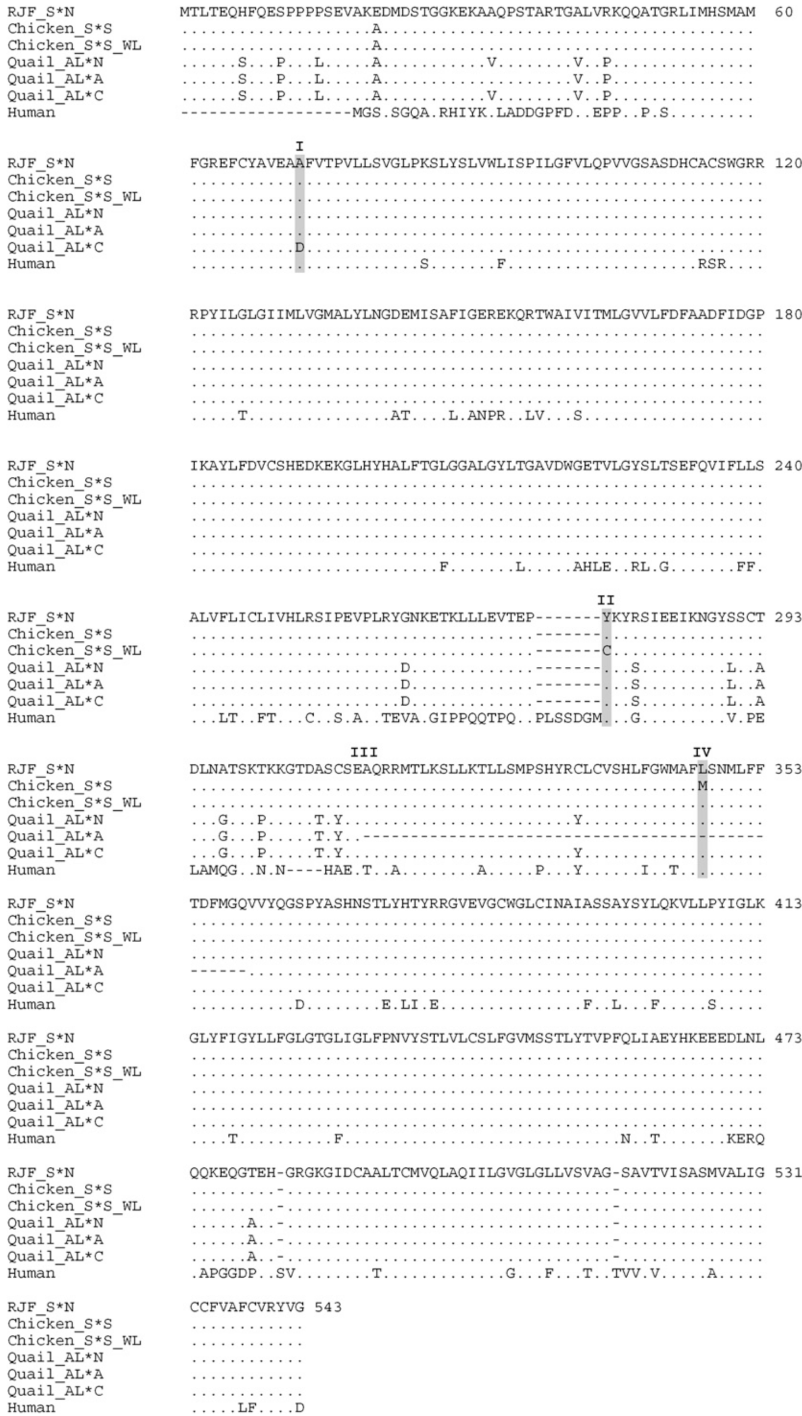

Figure 4.—Multiple-protein alignment of SLC45A2 using chicken, quail, and human sequences. (I) The quail A72D mutation associated with *cinnamon*. (II) The chicken Y277C mutation associated with *Silver* in White Leghorn. (III) The beginning of the region that is missing in *sex-linked imperfect albinism* in Japanese quail. (IV) The chicken L347M mutation associated with *Silver*. A dot indicates identity to the master sequence and a dash indicates insertion/deletion differences between the sequences.

White Leghorns are presumed to carry the *Silver* allele. The tested White Leghorns representing three different lines did not carry the L347M mutation but were homo- or hemizygous for the Y277C mutation. However, the data across populations did not provide an unequivocal support for this being a causative mutation since it was also common in domestic fowl assumed to be non-*Silver*, including the Rhode Island Red, which apparently expresses red pigmentation. This missense mutation was uncommon in the red jungle fowl, and only 1 of 13 birds carried this mutation (Table 1).

Weak association between Y277C and plumage color in a red jungle fowl/White Leghorn intercross: The extensive variation in plumage color in our intercross between the red jungle fowl and White Leghorn is expected to be controlled by at least four loci: *Dominant white*, *Extension*, *Silver*, and *Barred*. We have previously demonstrated that *Dominant white* in White Leghorns is due to a 9-bp insertion in the coding sequence of *PMEL17* (Kerje *et al.* 2004) and *Extended black* represents a missense mutation in the gene for the melanocortin 1-receptor (*MC1R*) (Kerje *et al.* 2003). Since the single

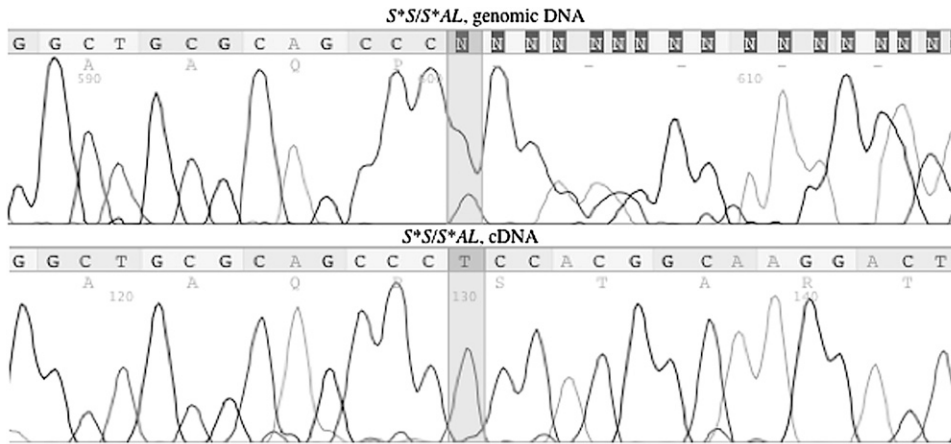

Figure 5.—Sequence traces for part of chicken *SLC45A2* exon 1 indicating nonsense-mediated mRNA decay (NMD) of the *S*AL* transcript. The top sequence shows sequenced genomic DNA of a chicken heterozygous for *106delT*; position 106 is heterozygous *T/delT* and the two sequences are out of frame after this position. The bottom sequence shows the cDNA sequence from the same individual; only the wild-type sequence is apparent.

red jungle fowl male was homozygous for the *SLC45A2 277Y* allele and the three White Leghorn founder females were hemizygous for *277C* we could utilize the data on plumage color from ∼800 F_2 progeny to investigate a possible association between this mutation and plumage color. No significant association was recognized in males (data not shown) while a weak, but significant, association was revealed in females (Table 2). The difference between sexes was expected since *277C* was present only in the heterozygous condition among males, due to the experimental design. It is well known that *Silver* is not fully expressed in heterozygotes (Smyth 1990). The observed association in females was to a large extent consistent with the expected effect of *Silver*, but the effect was not fully penetrant. Most birds expressing the reddish *Cream* phenotype were heterozygous for *Dominant white*, which primarily inhibits expression of black eumelanin, and the majority of these birds carried the wild-type *SLC45A2* allele (*277Y*), which should allow full expression of red pigmentation. However, as many as 13 of 41 birds expressed cream color despite being hemizygous for *277C* (Table 2). Similarly, a fraction of the birds expressing the wild-type color pattern lacked yellow/red pigmentation and were denoted grayish wild type. Twelve of 16 birds were hemizygous for *277C*, consistent with the expected effect of *Silver*, but the remaining 4 were hemizygous for the wild type, and hence the association was not complete. A small number of birds were classified as gray and they were, somewhat unexpectedly, all hemizygous for the wild-type *SLC45A2* allele. This could be due to the segregation of another feather color mutation, the *Blue* mutation, which is known to be present in White Leghorns (Smyth 1990). Heterozygous carriers of this mutation are blue–gray when they also carry the dominant black allele at the *E/MC1R* locus.

The incomplete association between *SLC45A2* and plumage color in this intercross may reflect incomplete penetrance because other genetic factors are suppressing the expression of the *SLC45A2* mutation. Another explanation is that the effect is due to a linked locus showing a fairly high recombination rate with *SLC45A2*. We tested the latter hypothesis by carrying out the same association analysis using the flanking markers *ADL0022* and *MCW0053* located 12.4 and 16.1 cM from *SLC45A2*, respectively. However, none of these markers showed a stronger association to variation in plumage color. This result suggests that the association is due to *SLC45A2* or another locus in the near vicinity showing an incomplete penetrance in regard to plumage color.

DISCUSSION

This article demonstrates that *SLC45A2* is the causative gene for the sex-linked *Silver* locus in chicken and *sex-linked imperfect albinism* in Japanese quail. Previous studies using intergeneric crosses demonstrated that matings between *albino* chicken and quails (*S*AL* and *AL*A*, respectively) produce only *albino* progeny and these alleles must thus be due to mutations in a homologous gene (Silversides and Mérat 1991). Our observation of disrupting mutations associated with both *albino* alleles, a frameshift mutation at codon 36 in chicken, and a splice-site mutation leading to exon skipping in the quail therefore provides conclusive evidence that *SLC45A2* is underlying these loci.

SLC45A2 transcription is thought to be indirectly regulated by the melanocyte-specific transcription factor MITF (Du and Fisher 2002) and *SLC45A2* ESTs have not been found in chicken brain cDNA libraries (http://www.genome.ucsc.edu). Surprisingly, we could amplify *SLC45A2* 5′ and 3′ cDNA sequences from a RACE-ready first-strand brain cDNA library. However, it is possible that *SLC45A2* is expressed at a low level in brain and that we could amplify this sequence due to the high sensitivity of the RT–PCR method.

Here we describe five different *SLC45A2* mutations associated with variation in plumage color in chicken and Japanese quail. Two of them are apparent null mutations causing *sex-linked imperfect albinism*. The deletion of 1 bp (106delT) in chicken *S*AL* exon 1 results

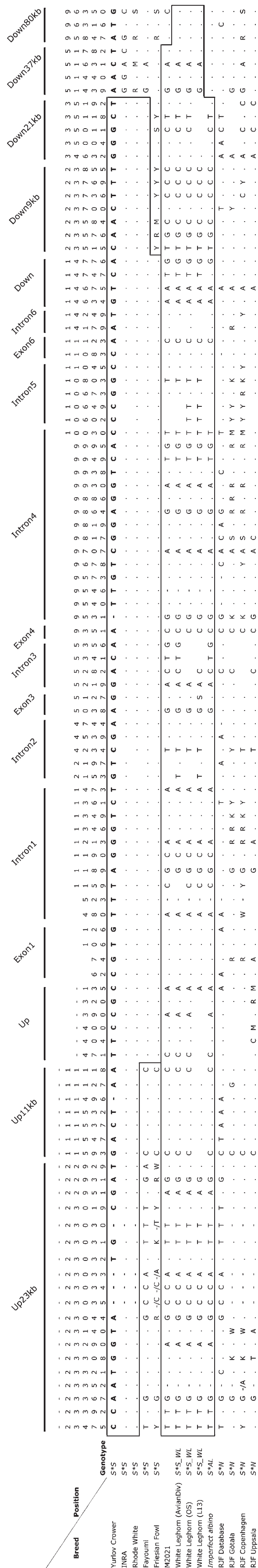

FIGURE 6.—Alignment of nucleotide sequence polymorphisms in *SLC45A2* and flanking regions among chickens with different genotypes at the *Silver* locus. All seven exons (1959 bp) were sequenced. Totals of 4081 bp of intronic sequence adjacent to the exons and 4128 bp of up- and downstream sequences were analyzed. The Yurlow Crower is used as the master sequence, dots indicate identity to the master sequence, dashes indicate insertion/deletion differences between the sequences, and open spaces indicate no information. Heterozygote positions are denoted as follows: R, A/G; Y, C/T; K, G/T; M, A/C; S, G/C; and W, A/T. The marked areas illustrate shared haplotypes between breeds. All sequences originate from breeds included in Table 1 except RJF Database and RJF Uppsala. RJF Database is the publicly available genome sequence of the chicken genome and RJF Uppsala is the breed used in our red jungle fowl/White Leghorn intercross.

in a frameshift and a premature stop codon and the transcript is apparently degraded by nonsense-mediated mRNA decay (Maquat 2005). Melanocytes from *S*AL* birds, *in situ* and in culture, have been found to have morphologically strange melanosomal/lysosomal organelles that are positive for both tyrosinase and acid phosphatase activities, and numerous morphologically normal premelanosomes were found to be lacking both enzymes (Boissy *et al.* 1987). The conclusion was that in the *S*AL* melanocyte, tyrosinase is not efficiently shuttled to the premelanosome. Since the normal function of *SLC45A2* is believed to be in directing tyrosinase to these premelanosomes (Costin *et al.* 2003), our finding of a loss-of-function mutation in *S*AL* individuals is fully consistent with the observed phenotype of the *S*AL* melanocyte.

The splice-site mutation in quail *albinos* results in an in-frame skipping of exon 4 (Figure 3) and 47 amino acids are missing in the mature protein, including transmembrane region 7. This also leads to a phase shift for the location of the remaining loop regions in relation to different sides of the membrane. Thus, it is not surprising that this mutation causes *imperfect albinism* in the quail. Previous studies on this mutant have revealed that the mutant melanocytes show tyrosinase activity in the Golgi–endoplasmic reticulum–lysosome (GERL) region and in the Golgi vesicles, but not in the eumelanosomes. The same study also concluded that the *AL* gene may affect the transport of tyrosinase from the GERL or Golgi vesicles to the melanosomes (Yamamoto *et al.* 1987), once again fully consistent with the known function of SLC45A2 (Costin *et al.* 2003). *Sex-linked imperfect albinism* has been found in several other avian species such as turkey, budgerigar, and canary (Hutt 1949). Furthermore, *sex-linked imperfect albinos* have appeared a number of times in different populations of chicken and quail, suggesting that there are many more *SLC45A2* mutations to be found in avian species.

The mutation (Ala72Asp) associated with *cinnamon* in Japanese quail was found in a highly conserved transmembrane region (Figures 3 and 4). This allele gives a more severe phenotype than *Silver* in chicken. The eyes of the chicks are red and have subnormal melanin pigmentation that darkens with age as in the *imperfect albino* birds (Cheng and Kimura 1990). The mutation results in a dilution of brown pigment in the feathers but the plumage pattern is not affected; the same ghost patterning is seen in *imperfect albinos*. The Ala72Asp mutation is a nonconservative amino acid substitution that may well explain the strong phenotypic effect of this missense mutation.

Two different missense mutations were associated with *Silver* in chicken. One of these (Leu347Met) was located in a highly conserved transmembrane region (Figures 3 and 4) and showed a complete association with the presence of *Silver* across breeds (Fayoumi, Yurlov Crower, Rhode White, Friesian Fowl, and White

TABLE 1

Distribution of the Y277C and L347M mutations in chicken *SLC45A2* across populations

		Y277C			L347M		
Breed (origin)	*S* allele	A/A, A/W (wt)	G/– (*S***S_WL*)	Total	C/C, C/W (wt)	A/– (*S***S*)	Total
Fayoumi (INRA, France)	*S***S*	5	0	5	0	5	5
Yurlov Crower (INRA, France)	*S***S*	5	0	5	0	5	5
Rhode White (AvianDiv)	*S***S*	8	0	8	0	8	8
Friesian Fowl (AvianDiv)	*S***S*	2	0	2	0	2	2
White Buttercup (Nutreco, Holland)	*S***S*	9	5	14	2	104	106
White Leghorn (AvianDiv)	*S***S_WL*	0	8	8	8	0	8
White Leghorn (SLU13, Sweden)	*S***S_WL*	0	15	15	16	0	16
White Leghorn (OS, Sweden)	*S***S_WL*	0	6	6	6	0	6
Brown Buttercup (Nutreco, Holland)	*S***N*	0	21	21	116	5	121
Godollo Nhx (AvianDiv)	*S***N*	1	7	8	8	0	8
Green-Legged Partridge (AvianDiv)	*S***N*	0	8	8	8	0	8
Rhode Island Red (AvianDiv)	*S***N*	1	7	8	8	0	8
Ukrainian Bearded (AvianDiv)	*S***N*	0	8	8	7	0	7
Friesian Fowl (AvianDiv)	*S***N*	4	1	5	6	0	6
Red jungle fowl (Ebeltoft, Denmark)	*S***N*	2	1	3	5	0	5
Red jungle fowl (Frösö, Sweden)	*S***N*	3	0	3	3	0	3
Red jungle fowl (Götala, Sweden)	*S***N*	5	0	5	5	0	5
Red jungle fowl (Copenhagen)	*S***N*	2	0	2	3	0	3
Total		47	87	134	201	129	330

Buttercup) with the exception of White Leghorn. We therefore postulate that this is a causative mutation for the Silver phenotype among chickens. Seven birds with conflicting genotypes were observed in a family material but we are convinced that these are due to phenotyping errors since the phenotyping was performed using 1-day-old chicks.

We could not obtain conclusive evidence that the Tyr277Cys mutation associated with *Silver* in White Leghorns is causative. This mutation was found in a number of birds that were not presumed to carry *Silver* (Godollo Nhx, Green-legged Partridge, Rhode Island Red, Ukrainian Bearded, birds from the Nutreco experimental cross, and one red jungle fowl), suggesting that this could rather be a linked polymorphism. Furthermore, the mutation occurs in a poorly conserved region of the protein, but it is a nonconservative substitution at a site that is conserved between chicken and humans. The following observations strongly suggest that White Leghorns carry an *SLC45A2* mutation: (i) all White Leghorns from three different lines were homozygous for the same *SLC45A2* haplotype; (ii) the size of this shared haplotype among different White Leghorn lines was >100 kb, strongly indicative of a selective sweep at this locus; and (iii) the *SLC45A2* segregation shows a weak, but significant, association to variation in plumage color in our red jungle fowl/White Leghorn intercross. Interestingly, the expression of red pigment most likely due to a leaky *Silver* allele has been observed as a problem in White Leghorn lines that can be corrected by selecting against the phenomenon (R. Okimoto, personal communication). This supports the notion that the *Silver* allele in the White Leghorn is not fully penetrant and its expression is influenced by the genetic background. Therefore, we consider Tyr277Cys as a candidate mutation for *Silver* in White Leghorns but it is also possible that *Silver* in White Leghorns is caused by a regulatory mutation located within the +100-kb haplotype shared by different strains of White Leghorn.

There has been strong selection at the *Silver* locus in chicken since the presence of *Silver* has been required

TABLE 2

Association between the *SLC45A2* Y277C missense mutation and variation in plumage color among female F_2 progeny in a red jungle fowl/White Leghorn intercross

	SLC45A2 genotype			Chi square
Phenotype	*277Y*/W	*277C*/W	Total	(d.f. = 1)
White[a]	79	84	163	0.1
Cream[a]	28	13	41	5.5*
White with black spots[a]	16	10	26	1.4
Black with white spots	6	2	8	2.0
Gray	5	0	5	5.0*
Black	9	6	15	0.6
Wild type, grayish	4	12	16	4.0*
Wild type, normal	4	6	10	0.4
Barred, gray	10	10	20	0
Barred, yellow	2	1	3	0.3

* $P < 0.05$.

[a] These birds were all homozygous or heterozygous for the *Dominant white* (*I*) allele, whereas all other classes were homozygous wild type (*i/i*).

for many breed-specific plumage patterns like the white color in White Leghorns. All breeds included in this study carrying *Silver* at present are expected to be fixed for this allele. Thus, the data presented in Figure 6 are fully consistent with this as the five divergent breeds carrying the *Silver* allele associated with the *SLC45A2 347M* mutation are homozygous for the same haplotype across *SLC45A2*. Similarly, the three different lines of White Leghorns are all homozygous for another haplotype carrying the *277C* mutation. In sharp contrast, an examination of the sequence data from three red jungle fowls representing different Scandinavian zoo populations plus the genome sequence based on a single red jungle fowl bird reveals as many as 31 polymorphic sites in *SLC45A2* (Figure 6). This marked difference in the degree of polymorphism between populations is a hallmark of a selective sweep for a favorable mutation (Maynard-Smith and Haigh 1974; Andersson and Georges 2004). The minimum shared haplotype among all chicken breeds carrying the *347M* allele, firmly associated with *Silver*, was in the range 15–35 kb. This suggests that a marker density of one SNP/10 kb would have been sufficient to identify the *Silver* locus by a genomewide association analysis across breeds. The minimum shared haplotype among different White Leghorn lines, presumed to carry a second *Silver* allele, was even larger (>100 kb). This illustrates that the SNP density required for a genomewide association analysis will depend on the size affected by a selective sweep and the genetic distance among populations carrying the same mutation.

The most interesting feature of the *SLC45A2* mutations described here is the specific inhibition of the expression of red pheomelanin in birds carrying *Silver*. This phenotypic effect is well documented in previous ultrastructural characterization of melanosomes from *Silver* and non-*Silver* birds (Brumbaugh 1971). A dilution of red pigment with no or only a minor effect on black pigment is also observed in horses heterozygous for a D153N mutation in *SLC45A2* (Mariat *et al.* 2003). Horses homozygous for this mutation, though, have blue eyes and very little coat pigmentation as a result of a considerable dilution of both red and black pigments. In contrast, *Silver* in chicken has no effect on black pigment even in the homozygous condition. The specific inhibition of the production of pheomelanin that occurs in chickens carrying the *Silver* allele and in horses that are heterozygous for the D153N mutation cannot be explained by the current understanding of SLC45A2 function.

The amino acid cysteine is essential for the synthesis of red pigment whereas very little tyrosinase activity is required. It is therefore tempting to speculate that one of the functions of SLC45A2 is to transport cysteine into the melanosome and that this function is disrupted by the mutations associated with *Silver*. In previous studies it has been suggested that *Silver* may block the incorporation of sulfhydryls in pheomelanin since ultrastructural studies showed that sparsely melanized pheomelanosomes were coincident with histochemically sulfhydryl-negative melanocytes (Brumbaugh 1971). The *subtle gray* (*sut*) mouse has a mutation in the *Slc7a11* gene encoding the cystine/glutamate exchanger xCT (Chintala *et al.* 2005). xCT has 12 predicted transmembrane regions and the cystine taken up by the cell via xCT is rapidly reduced to cysteine (Sato *et al.* 1999). An abnormal accumulation of tyrosinase (possibly in the trans-Golgi network) was found in the *sut* melanocyte, showing an anomalous trafficking of tyrosinase in the absence of cysteine. This abnormality does not seem to have any affect on eumelanin production (Chintala *et al.* 2005). SLC45A2 is also a 12-transmembrane transport protein but it is still unclear which molecules it transports (Fukamachi *et al.* 2001). The phenotypic effects of *Silver* in chicken and the D153N mutation in horse suggest that SLC45A2 may be crucial for the intracellular transport or melanosome content of cysteine after the xCT exchanger has allowed cystine to enter the melanocytes. This cysteine could be essential for the transport vesicles that direct tyrosinase from the trans-Golgi network to the pheomelanosome, and therefore mutations in *Slc7a11* and some *SLC45A2* mutations are affecting only pheomelanin production. There are also other mutations found to affect pheomelanin production. The *gray-lethal* (*gl*) mouse mutation in the *GL/OSTM1* gene results from stalled pheomelanin migration due to clustered pheomelanin granules (Chalhoub *et al.* 2003), and the γ-glutamyl transpeptidase (*GGT*) knockout mice lack cysteine as a result of the nonworking cleavage of glutathione (Lieberman *et al.* 1996). However, if SLC45A2 has an important role for the incorporation of sulfhydryls into pheomelanin it cannot be its sole function since a defect in cystine/cysteine transport should not affect eumelanogenesis, whereas *SLC45A2* null mutations almost completely abolish the production of both eumelanin and pheomelanin. It is an enigma why recessive null mutations cause an almost complete absence of both eumelanin and pheomelanin, whereas some missense mutations are dominant and cause a specific inhibition of the expression of red pheomelanin. Therefore, further experimental work based on the missense mutations in chicken and horse may shed light on SLC45A2 function.

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