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Characterization of Japanese Quail *yellow* as a Genomic Deletion Upstream of the Avian Homolog of the Mammalian *ASIP* (*agouti*) Gene

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ABSTRACT

ASIP is an important pigmentation gene responsible for dorsoventral and hair-cycle-specific melanin-based color patterning in mammals. We report some of the first evidence that the avian *ASIP* gene has a role in pigmentation. We have characterized the genetic basis of the homozygous lethal Japanese quail *yellow* mutation as a >90-kb deletion upstream of *ASIP*. This deletion encompasses almost the entire coding sequence of two upstream loci, *RALY* and *EIF2B*, and places *ASIP* expression under control of the *RALY* promoter, leading to the presence of a novel transcript. *ASIP* mRNA expression was upregulated in many tissues in *yellow* compared to wild type but was not universal, and consistent differences were not observed among skins of *yellow* and wild-type quail. In a microarray analysis on developing feather buds, the locus with the largest downregulation in *yellow* quail was *SLC24A5*, implying that it is regulated by *ASIP*. Finally, we document the presence of ventral skin-specific isoforms of *ASIP* mRNA in both wild-type quails and chickens. Overall, there are remarkable similarities between *yellow* in quail and *lethal yellow* in mouse, which involve a deletion in a similar genomic position. The presence of ventral-specific *ASIP* expression in birds shows that this feature is conserved across vertebrates.

THE agouti signaling protein (ASIP) encoded at the *ASIP/agouti/ASP* locus is a well-characterized component of the mammalian melanocortin system. Its primary function is as an endogenous inverse agonist of the melanocortin-1 receptor (MC1R) in hair follicle melanocytes with expression decreasing eumelanin (dark black/brown pigment) and increasing pheomelanin (pale yellow/red pigment) production (Gantz and Fong 2003). Many *agouti* mutations are known in mice and these can largely be grouped into dominant, gain-of-function mutations causing a pale phenotype (*e.g.*, A^{y}, A^{vy}) and recessive, loss-of-function mutations causing a dark phenotype (*e.g.*, *a*, a^{t}). Four different *agouti* mRNA isoforms are present in wild-type mice, produced by differential transcription of four different noncoding exons (1A, 1A′, 1B, and 1C) (Siracusa 1994; Vrieling *et al.* 1994; Millar *et al.* 1995). Two of these (1A and 1A′) are expressed only in the ventral skin of wild-type mice, producing a pale-bellied phenotype (Chen *et al.* 1996), while the others (1B and 1C) are expressed in a temporal-specific manner during the hair-growth cycle producing banded or *agouti* hairs (Bultman *et al.* 1992).

The genetics of avian plumage color are of evolutionary interest because of the important role of coloration in signaling and mate choice (Andersson 1994; Hill and McGraw 2006), and some recent progress has been made in linking genetic changes to evolution of plumage coloration (Theron *et al.* 2001; Mundy *et al.* 2004; Nadeau *et al.* 2007a). However, our basic understanding of the pigmentation genetics of birds has lagged behind that of mammals. The presence of a functional *ASIP* gene in birds has been widely dismissed (Boswell and Takeuchi 2005). This is partly due to failed attempts to clone *ASIP* in chicken and the finding of peripheral expression of *AGRP*, an *ASIP* paralog expressed only in the nervous system in mammals, which was hypothesized to take the role of *ASIP* during melanogenesis (Takeuchi *et al.* 2000). However, an *ASIP*-like sequence was recently reported to be present on chicken chromosome 20 (GGA20) (Klovins and Schiöth 2005), warranting further investigation.

The *yellow* mutation (*Y*) of Japanese quail (*Coturnix japonica*) is an autosomal dominant mutation with homozygous (*Y/Y*) lethality. Heterozygotes (Y/y^{+}) have wheat-straw yellow-colored feathers (Figure 1) and

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Figure 1.—Male Japanese quail (*Coturnix japonica*) of *yellow* (Y/y^+) (left) and wild-type (y^+/y^+) (right) phenotypes.

abnormal metabolism, including higher levels of abdominal fat (Minvielle *et al.* 2007). The effects of this mutation, therefore, show some similarities to the mouse *lethal yellow* (A^y) mutation, which is due to a 170-kb deletion upstream of the mouse agouti signaling-protein gene (*agouti*). This deletes the coding region of the *Raly* gene and puts *agouti* under control of the *Raly* promoter (Michaud *et al.* 1994). A^y causes ubiquitous expression of *agouti*, which produces the yellow coat color, and also causes several pleiotropic effects including obesity, diabetes, and tumor susceptibility. These wide-ranging effects can largely be accounted for by ASIP antagonism of several melanocortin receptor subtypes in addition to MC1R. Obesity of *yellow* mice can largely be explained by ASIP antagonism of MC3R and MC4R in the hypothalamus, which are normally antagonized by agouti-related protein (AGRP) to regulate feeding behavior and metabolism, although there is some evidence that ASIP in *yellow* mice may also act on MC2R within the adipocytes themselves (Miltenberger *et al.* 1997; Gantz and Fong 2003).

Avian homologs of all five mammalian melanocortin receptor subtypes have been identified. These are activated by the melanocortin peptides [primarily adrenocorticotropic hormone (ACTH) and α-MSH], which are synthesized locally from a common precursor, POMC (Ling *et al.* 2004). As in mammals, MC1R has a well-documented role in avian pigmentation and this appears to be its sole function (Takeuchi *et al.* 1996; Mundy 2005). MC4R and MC5R are both expressed in the brain as well as in several peripheral tissues and within the brain MC4R is involved in regulating feeding behavior (Takeuchi and Takahashi 1998; Strader *et al.* 2003). Although AGRP has widespread expression in the chicken, its expression in the avian brain shows a clear relation to feeding behavior similar to that found in mammals (Boswell *et al.* 2002). As in mammals, avian MC2R appears to be primarily involved in mediating the effects of ACTH on biogenesis of corticosteroids in the adrenal gland (Ling *et al.* 2004). However, chicken MC3R also has adrenal-specific expression and is not found in the brain (Takeuchi and Takahashi 1999).

Previous evidence that *Y* may be a mutation of avian *ASIP* has come from a study that mapped *Y* to the quail chromosome homologous to chicken chromosome 20 (GGA20) (Miwa *et al.* 2005). In addition, crossing experiments between *extended brown* and *yellow* quails indicate that *E* is epistatic to *Y*—*i.e.*, the phenotypic effects of *yellow* are masked in *extended brown* individuals (Somes 1979; F. Minvielle, unpublished results). Therefore, *yellow* acts upstream of *MC1R* (*extended brown*, Nadeau *et al.* 2006), which is consistent with the epistasis seen between *Mc1r* (*extension*) and *agouti* in mice. If *Y* is a mutation of *ASIP*, it will be the first evidence that this locus is functional and plays a role in pigmentation in birds.

MATERIALS AND METHODS

Quail samples: All Japanese quail (and chickens) were maintained at the INRA Experimental Unit GFA in Nouzilly (France). *Yellow* quail were from a line established in Gifu University, Japan and maintained in Nouzilly. Single-pair matings between these and wild-type birds were carried out to obtain three families segregating for the *yellow* mutation. Six males of each phenotype were sampled from each of these families. Skin samples were taken by dissecting a piece of skin (~4 cm^2), which was either snap frozen in liquid nitrogen or immersed in RNAlater (Ambion, Austin, TX). Two of these families had feathers plucked from the region of skin that would be sampled 11 days prior to sampling, to stimulate feather growth. Dorsal skin samples were taken from the unplucked and one of the plucked families. Dorsal and ventral skin and several other tissues (including brain) were sampled on a single day from the third family. These individuals were all killed in the morning and were in a fed state. Skin was also sampled from dorsal and ventral regions of six more male wild-type quail. All skin and organ samples were taken from adult quail (at least 6 weeks old, see supplemental Table 1 at

http://www.genetics.org/supplemental/ for further details). All dorsal skin samples were taken from the region overlying the pelvis at the level of the ilium and all ventral samples were from the region over the pectoral muscles.

Total RNA was extracted from the skin samples using the RNeasy mini-kit (QIAGEN, Valencia, CA). RNA concentration, purity, and integrity (RIN values) were checked using a BioAnalyzer (Agilent). RNA was stored at −80° until use. cDNA syntheses were performed in a 20-μl volume with 1–3 μg total RNA and 150 ng/μl N6 primer using Superscript RT II (Invitrogen, San Diego) and following the manufacturer's instructions.

Matings were also carried out between two *yellow* pairs to obtain two further families segregating for the *yellow* mutation. Genomic DNA was extracted from the parents and all offspring (22 from one family and 20 from the other), from blood using standard methods.

Sequencing the *ASIP* coding region: cDNA from two of the families segregating for *yellow* was used to amplify 361–384 bp of the 393-bp coding region of *ASIP*, using primers ASIPF2 or ASIPF5 and ASIPR5 (see supplemental Table 2 at http://www.genetics.org/supplemental/ for primer sequences) designed on the basis of the chicken mRNA sequence (Klovins and Schiöth 2005). PCRs were performed in a 50-μl total reaction containing 1.0 unit Taq polymerase (Advanced Biotechnologies, London), 1× reaction buffer, 1.5 mm $MgCl_2$, 50 mm each dNTP, 10 nm each primer, and 2–4 μl of product from the cDNA reactions. PCR reactions were performed in a DNA Engine (MJ Research, Watertown, MA), with the following cycling parameters: 94° for 2 min; 94° for 30 sec, 55°–60° for 45 sec, 72° for 1 min 40–45 times; and 72° for 5 min. PCR products were directly sequenced on both strands using the PCR primers.

Sequencing of 5′-noncoding regions: 5′-noncoding regions identified from EST data in the chicken genome (v1.0) were amplified using forward primers ASIPF6, ASIPF7, and ASIPF8 designed to the predicted noncoding regions (which we hereafter refer to as exons 1a, 1b, and 1c, respectively) and a reverse primer, ASIPR6, within the coding region. Forty to 45 cycles of PCR were performed as described above. PCR products were run on a 1% agarose gel and directly sequenced on both strands using the PCR primers.

The relative positions of exons 1b and 1c were confirmed by long-range PCR, using primers ASIPF7 and ASIPR13 with Extensor Hi-Fidelity PCR master mix (ABgene). This generated a product of ~6 kb, the identity of which was confirmed by direct sequencing of the ends using the PCR primers.

To identify the 5′-noncoding regions associated with the *Y* allele, 5′ rapid amplification of cDNA ends (RACE) was performed on a *yellow* individual and on a wild-type sibling as a control. The Invitrogen 5′ RACE system, version 2.0, was used according to the manufacturer's instructions. The gene-specific primer used for cDNA synthesis was ASIPR1. The second gene-specific primer, which was used for the first round of PCR amplification, was ASIPR6. Further gene-specific primers, ASIPR7 and ASIPR9, designed to bind only to the *Y* allele, were then used to perform secondary PCRs. Products were then directly sequenced using the PCR primers and the novel region of noncoding sequence was compared to the chicken genome using a BLAST search. The presence of this transcript in only the *yellow* samples was then confirmed by a PCR using the original cDNA samples from the *yellow* and non-*yellow* dorsal and ventral samples with primers ASIPF10 and ASIPR1.

Sequencing of markers upstream of *ASIP*: Five genomic regions upstream of *ASIP* were sequenced and found to contain variation in the parental alleles of one of the families sampled for genomic DNA. All 20 offspring from these parents were then amplified and sequenced for these regions. The second set of parents sampled did not contain variation in these regions and therefore this family was not analyzed further. The first region was 506 bp within intron 1 of the *RALY* gene, which was amplified with primers RALYF2 and RALYR3 and sequenced with these and an internal sequencing primer, RALYR4. The second was a 998-bp region spanning intron 4 of *RALY*, amplified with primers RALYF1 and RALYR1 and sequenced with these and internal sequencing primers RALYF4, RALYF5, RALYR2, and RALYR5. Third, a 1-kb region spanning intron 2 of the *EIF2B* gene was amplified using primers EIFF1 and EIFR2 and sequenced with these and internal sequencing primers EIFF2, EIFR3, and EIFR4. Fourth, a 303-bp region within intron 1 of the *EIF2B* gene was amplified and sequenced with primers Gg20MSB2F and Gg20MSB2R. Finally, a 527-bp region within the intron between exons 1b and 1c of *ASIP* was amplified and sequenced with primers ASIPF21 and ASIPR13. PCR reactions were performed as described above with 35 cycles and 50–200 ng of genomic DNA.

Quantitative RT–PCR of *ASIP* in skin: Quantitative RT–PCR was used to investigate *ASIP* expression in the dorsal and ventral skin of the six wild-type quails and in the skin of the three families segregating for *yellow*. Quantitative RT–PCR was performed for *ASIP* using primers ASIPF1 and ASIPR1, which amplified a 338-bp product within the coding region. Reactions were performed in a 25-μl total reaction containing 1× SYBR Green master mix (QIAGEN), 10 nm each primer, and 1–2.5 μl of product from the cDNA reactions. Reactions were performed in an Opticon 2 DNA engine (MJ Research), with the following cycling parameters: 95° for 15 min; 94° for 15 sec, 55°–58° for 30 sec, 72° for 30 sec 40–55 times; and 72° for 10 min. Melting curves were generated between 55° and 90° with readings taken every 0.2° for each of the products to check that a single product was generated. At least one product from each set of primers was also run on a 1% agarose gel to check that a single product of the expected size was produced and the identity of the product was confirmed by direct sequencing. Two housekeeping genes were used for normalization: β-*actin* and *GAPDH* were amplified with primers ACTF1, ACTR1, GAPDHF1, and SJ2, generating products of 258 and 249 bp, respectively. Amplified fragments always spanned at least one intron to ensure that genomic DNA contamination could be identified.

C_t values were defined as the point at which fluorescence crossed a threshold (R_{C_t}) of 10× standard deviation (SD) of the background fluorescence. Amplification efficiencies (E) were calculated using a dilution series of clean PCR product. Starting fluorescence, which is proportional to the starting template quantity, was calculated as $R_0 = R_{C_t}(1+E)^{-C_t}$. Normalized values were then obtained by dividing R_0 values for the target loci by R_0 values for β-*actin* or *GAPDH*. All results were taken as averages of triplicate PCR reactions and PCRs on target and control loci were always performed using product from the same cDNA synthesis reaction. Statistical significance was assessed using an unrelated samples two-tailed *t*-test assuming unequal variance for the *yellow*/wild-type samples or a Wilcoxon signed-rank test for the paired dorsal/ventral wild-type samples.

RT–PCR of *ASIP* in tissues: PCR for *ASIP* was performed on cDNAs from plucked dorsal and ventral skin, uropygial gland, spleen, testis, brain, heart, liver, adrenal gland, skeletal muscle, and kidney from two wild-type and *yellow* individuals from one of the families. Reactions were performed with 0.1 μg of total RNA per reaction, primers ASIPF1 and ASIPR1, and 45 cycles of PCR. *GAPDH* was used as a control to check for cDNA synthesis and was amplified as described above using 40 cycles of PCR.

Microarray analysis of gene expression in *yellow* skin: cDNAs were prepared and amplified using the SMART kit (Clontech, Palo Alto, CA) from total RNA from the plucked

dorsal skin of six *yellow* and wild-type male quails from one of the families. Amplified cDNAs were labeled using the BioPrime Plus Array CGH genomic labeling system (Invitrogen Life Technologies). The cDNAs from the wild-type individuals were labeled using fluorochrome *Cy3*, and the *yellow* individuals were labeled with *Cy5* with the dyes swapped on three of the six pairs of samples. Microarrays were constructed using a library of 20,460 60mer–75mer 5′-amine-modified oligo probes (Operon and Roslin/ARK CoRe chicken array set). The labeled cDNAs were concentrated by precipitation and resuspended in 60 μl hybridization buffer (18.5% formamide, 5× SSC, 5× Denhardt's solution, 0.5% SDS, 5 mm KH_2PO_4), denatured at 95° for 5 min, and hybridized to the array at 42° overnight. Hybridized microarrays were scanned on a GenePix 4000B microarray scanner (Molecular Devices, Menlo Park, CA) and the resulting images were analyzed with GenePix array analysis software. Data analysis was performed in Acuity (Molecular Dynamics, Sunnyvale, CA). *Cy3*/*Cy5* ratios were $\log_2$ transformed and normalized by global lowess. Data were filtered by removing spots with <55% of pixels 1 SD above background and spots with a sum of medians <500 after subtracting median background values. Probes that failed the filtering criteria in more than one array were removed from the analysis. The significance analysis of microarrays (SAM) algorithm (Tusher *et al.* 2001) was used to identify significant differences in expression between *yellow* and wild-type individuals, using a false discovery rate (FDR) cutoff of 5%.

The result for *SLC24A5* was confirmed by quantitative RT–PCR on RNAs from the 12 *yellow* and wild-type individuals that were used for the array, performed as described above, with primers SLC24F2 and SLC24R2.

***ASIP* expression in chicken:** Dorsal and ventral skin samples were taken from 10 chickens, 2 males with a mottled phenotype and 8 females with a Columbian phenotype. cDNA was synthesized using the Ambion RETROScript kit from 2 μg of total RNA extracted using TRIzol (Invitrogen) followed by cleanup with the RNeasy mini-kit (QIAGEN). 5′-noncoding regions were amplified and sequenced as described above. In addition, semiquantitative RT–PCRs were carried out in a 50-μl volume with 1 μl from the cDNA reactions. Reactions for *ASIP* were carried out using primers ASIPF1 and ASIPR1 with 30 cycles and for *GAPDH* with primers SJ1 and SJ2 with 23 cycles of RT–PCR. Ten microliters of each reaction were run on a 1.5% agarose gel stained with SYBR-safe and quantified using GeneTools (SynGene), with relative expression inferred by normalization to *GAPDH* in each sample.

RESULTS

***ASIP* coding sequence in Japanese quail:** An amplicon of the predicted length was obtained by RT–PCR of *ASIP* from quail skin. The sequenced fragment was identical in length and had 95% similarity to the chicken *ASIP* coding sequence predicted by Klovins and Schiöth (2005), confirming the predicted coding exons and suggesting that this sequence does indeed encode an avian *ASIP* gene that is expressed in the skin. In addition to the predicted start codon, which gives the greatest homology to other *ASIP*s, an alternative in-frame start codon was also found 27 bp upstream (Figure 2). This end of the ASIP protein acts as a signal peptide mediating secretion. To investigate what effect the alternative start codon would have on peptide secretion, if it was functional, this region of sequence with or without the extra nine amino acids was analyzed in the program SignalP 3.0 (Nielsen *et al.* 1997; Bendtsen *et al.* 2004). This revealed that the predicted start codon produced a better signal peptide than the alternative upstream start codon ($S = 0.75$ and $S = 0.64$, respectively). It therefore seems likely that the predicted start codon is the functional one.

Sequencing of almost the entire coding region of *ASIP* in the two families segregating for the *yellow* mutation revealed two variable nucleotide positions (30 and 99), both of which were synonymous. These positions appeared to be in complete linkage disequilibrium with all alleles either being C30T99 or T30C99. In addition, the *yellow* phenotype was associated with the T30C99 allele ($P = 0.003$, Fisher's exact test), with all *yellow* individuals having at least one of these alleles. This would be consistent with the *Y* allele (which is present in a single copy in the *yellow* individuals) being linked to a T30C99 *ASIP* allele, while the y^+ allele can be T30C99 or C30T99 in composition. Together, these results suggest that the *yellow* mutation is not due to coding sequence variation at *ASIP* but is due to a mutation closely linked to this region.

5′-noncoding exons and identification of abnormal transcript in *yellow*: PCRs using primers designed to three potential 5′-noncoding exons all produced amplicons. Two of these, which we refer to as exon 1a and exon 1c, were amplified from both dorsal and ventral samples. Sequencing of these amplicons (Figure 2) revealed that beyond the primer sequence exon 1a was identical to 1c and therefore 1a may not represent a different isoform. Exon 1b was amplified strongly only from the ventral samples (Figure 2B) and sequencing revealed that this was a different isoform. Each of the noncoding exons was spliced directly onto exon 2, which contains the start codon.

We found the same pattern of expression of these noncoding exons in both the *yellow* and the wild-type individuals. However, sequencing of isoforms containing exons 1a and 1c from the *yellow* individuals from family 1 revealed that these transcripts contained only the C30T99 allele, which had been found to be associated with the y^+ allele. Similar results were not found for wild-type individuals containing both C30T99 and T30C99 alleles. Therefore, it appears that the *yellow* mutation causes a change in the 5′-untranslated region (UTR) of *ASIP* and that the *Y* allele does not contain the normally occurring 5′-noncoding exons. This would be consistent with the *yellow* mutation being due to a change in *ASIP* gene regulation. 5′-RACE revealed a novel 5′-noncoding region associated with the *Y* allele, which matched part of the predicted 5′-UTR of the chicken *RALY* gene, and this region shows reasonable similarity to the 5′-UTR of the mouse *Raly* gene. Therefore, it seems possible that the *yellow* mutation is a large deletion removing the *RALY* gene, placing *ASIP* under the control of the *RALY* promoter and causing a *RALY*

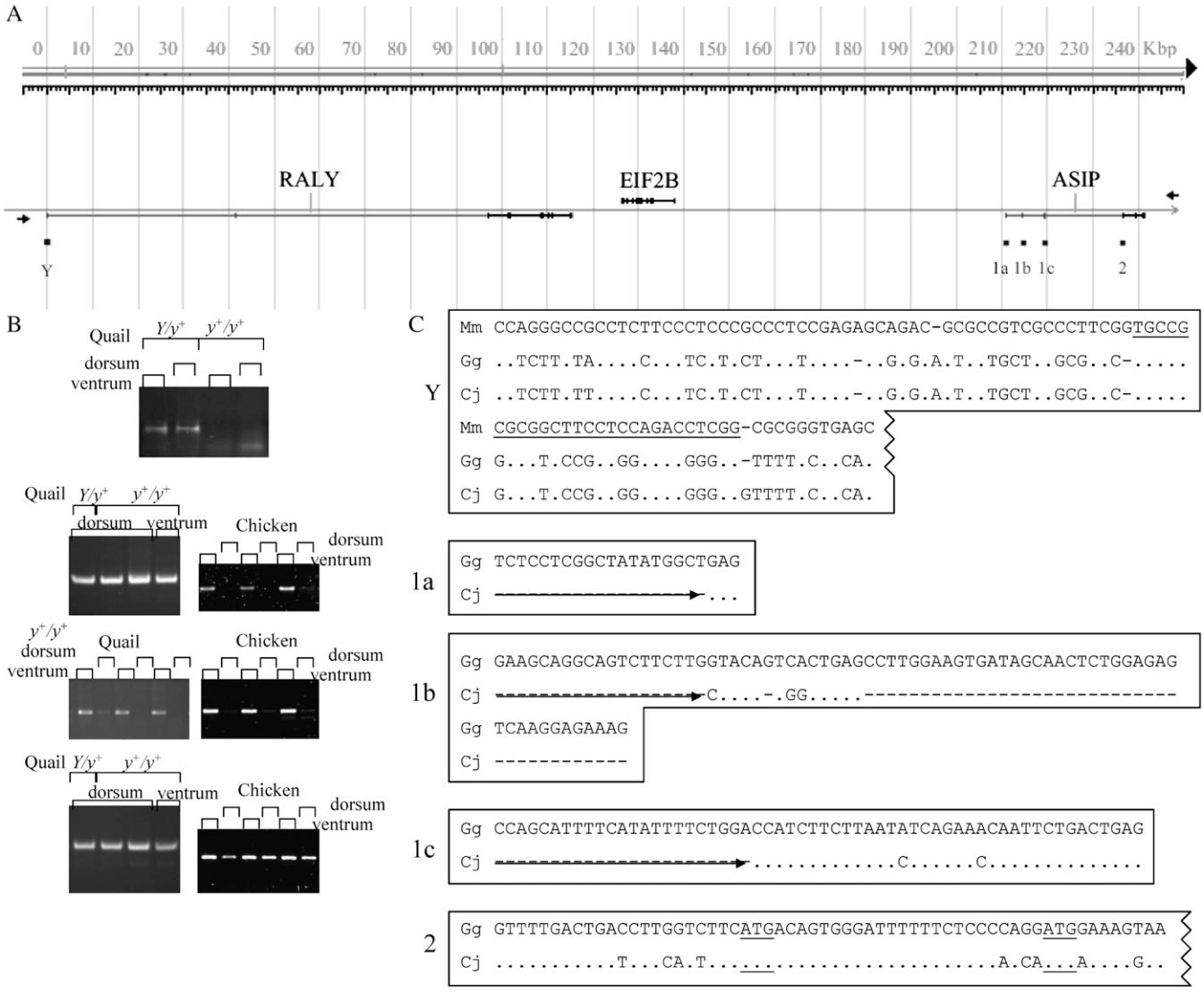
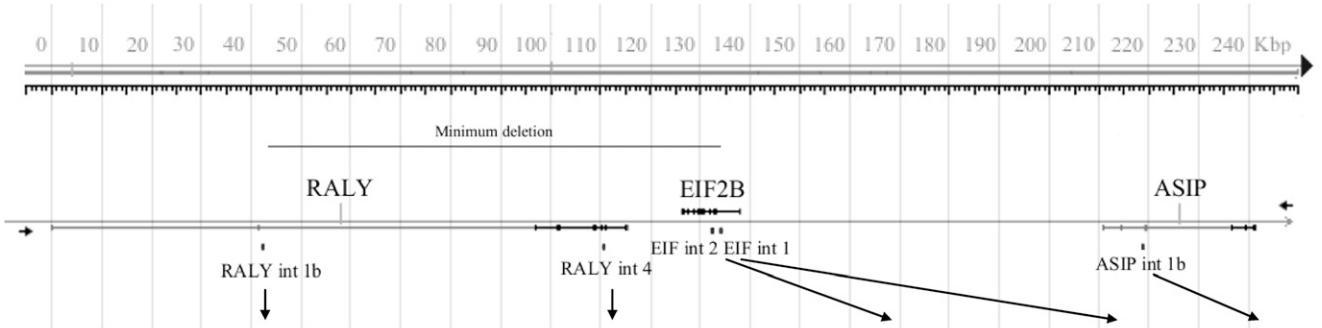


FIGURE 2.—Positions, nucleotide sequences, and expression patterns of the three avian *ASIP* noncoding exons and the 5′-noncoding sequence associated with the *Y* allele. (A) The positions of the *ASIP* exons and sequenced region associated with the *ASIP Y* allele (annotated as Y) in relation to the *RALY* gene on chicken chromosome 20. Solid exons, coding; shaded exons, noncoding; arrows indicate direction of transcription for the genes above (right) and below (left) the line. (B) mRNA expression, detected by RT–PCR, of each of the noncoding exons and the Y transcript, in yellow (Y/y^+) and wild-type (y^+/y^+) dorsal and ventral Japanese quail skin samples and dorsal and ventral chicken skin samples, amplified using forward primers as indicated in C and a reverse primer within the coding region of *ASIP* (see text for details). (C) Sequences of each of the 5′-UTRs with the chicken (Gg) EST sequences (accession nos. BU206868/BU337545) from which the primers were designed (marked with arrows) aligned to the quail (Cj) sequences. Within the alignments, missing nucleotides are indicated by dashes (–); dots (.) indicate sequence identity. For exon 1b the putative splice site in the chicken is beyond that identified in the quail. The 5′ portion of exon 2 is also shown with the two possible start codons underlined. The Y region found in the *yellow* quails is aligned to the homologous region of chicken chromosome 20 and to the 5′-noncoding exon of the mouse (Mm) *Raly* gene (accession no. NM_023130). The underlined region shows the most similarity between mouse and chicken/quail with 63% nucleotide identity.

noncoding exon to be found at the 5′ end of the *ASIP* gene.

Inheritance of linked markers: To determine if a deletion had occurred between the 5′-UTR of *RALY* and *ASIP* and to get an indication of its size, we developed markers in five genomic regions across this area and investigated their inheritance in $Y/y^+ \times Y/y^+$ matings. Two *yellow* (Y/y^+) parents showed homozygous differences for several SNPs and indels within four of these regions (Figure 3). Therefore, the null expectation was that all offspring should be heterozygous for these sites. Instead we found that all Y/y^+ offspring were homozygous for one or other of the parental alleles with only the y^+/y^+ offspring being heterozygous at all positions, and furthermore, all sites were in complete linkage disequilibrium in all Y/y^+ offspring, confirming their linkage in the quail genome. By far the simplest explanation for these results is that Y/y^+ individuals have a



	RALY intron 1b									RALY intron 4						EIF intron 2			EIF intron 1		ASIP intron 1b
	69	74	111	151	159	166	216	442-4	438	14	74	367	387	401	432	481	558	927-934	125	152	113
♀ Y/y+	T	G	C	C	G	C	G	Δ3	G	G	A	G	C	T	C	G	T	ACATCTGT	G	C	C/T
♂ Y/y+	C	A	T	T	T	T	T	GAG	A	A	G	Δ	A	C	T	A	G	Δ8	A	A	C/T
y+/y+ (4)	C/T	G/A	C/T	C/T	G/T	C/T	G/T	GAG/Δ3	*ns*	G/A	A/G	G/Δ	C/A	T/C	C/T	G/A	T/G	ACATCTGT/Δ8	G/A	C/A	C
Y/y+ (10)	T	G	C	C	G	C	G	Δ3	G	G	A	G	C	T	C	G	T	ACATCTGT	G	C	C/T
Y/y+ (6)	C	A	T	T	T	T	T	GAG	A	A	G	Δ	A	C	T	A	G	Δ8	A	A	C/T

Figure 3.—Genotypes of two *yellow* quail parents (Y/y^+ ♀ and ♂) and their 20 progeny (4 wild-type, y^+/y^+ and 16 *yellow* Y/y^+) at variable positions within five linked segments. Positions of these segments in the chicken genome are shown at the top. Four of the segments show evidence for a deletion linked to the *Y* allele, with all y^+/y^+ offspring being heterozygous at all positions and all Y/y^+ offspring showing only the maternal (10) or paternal (6) alleles. The fifth locus (*ASIP* intron 1b) is not deleted but shows evidence of linkage to the *Y* allele, with all Y/y^+ individuals being heterozygous and all y^+/y^+ individuals being homozygous. Δ*n*, number of nucleotides absent; ns, not sequenced.

deletion spanning all four of these regions on the *yellow* chromosome, and they have inherited a single wild-type copy from their parents. Within the fifth region, which was within the intron between *ASIP* exons 1b and 1c, the parental genotypes were identical and shared a single heterozygous site. Inheritance at this position was consistent with linkage to the *Y* allele, with all *yellow* individuals being heterozygous, C/T, and all wild-type individuals being homozygous for C. This also indicates that this region lies outside of the deletion. Assuming this area of the genome is a similar size between the quail and the chicken, this puts the size of the deletion at between 90 and 215 kb, spanning the entire coding sequence of *RALY* and at least 90% of the coding sequence of *EIF2B*.

***ASIP* expression in Japanese quail tissues:** Comparison of *ASIP* expression revealed significantly higher expression in the ventral than the dorsal wild-type skin samples (Table 1) ($P = 0.036$, Wilcoxon's signed-rank test), although there was extremely high variability in the extent of upregulation of ASIP in ventral as compared to dorsal skin between individuals. No significant difference was found between the *yellow* and the wild-type individuals in the plucked, unplucked, dorsal, or ventral skin samples from any of the three families (Table 2).

ASIP expression in the wild-type tissues was the highest in the skin and spleen, with weak expression in the adrenal gland, inconsistent expression between individuals in the uropygial gland, testis, and brain, and no expression in the heart, liver, skeletal muscle, or kidney (Figure 4). The *yellow* tissues generally appeared to have higher expression, particularly the adrenal gland, spleen,

TABLE 1

Relative expression of *ASIP* in ventral as compared to dorsal skin of six wild-type Japanese quail

	Relative expression (ventral/dorsal)
1	11.6
2	265.8
3	2.2
4	43.2
5	22.4
6	31.2

Expression is from quantitative RT–PCR normalized to β-*actin*.

TABLE 2

Relative expression of *ASIP* in *yellow* as compared to wild-type Japanese quail skin

Family	Skin sample	Mean relative expression (*yellow*/wt)	*P*-value
1	Unplucked dorsal (6/5)	19.17 ± 24.99	0.14
2	Plucked dorsal (6/6)	0.88 ± 0.86	0.84
3	Plucked dorsal (6/6)	0.83 ± 0.68	0.77
3	Plucked ventral (6/6)	0.51 ± 0.69	0.48

Expression is from quantitative RT–PCR, normalized to β-*actin* and relative to the mean of the wild-type samples in each set ± standard deviations. Samples were taken from three different families of *yellow*/wild-type quail. (*n*/*n*), *n yellow*/*n* wild type. *P*-values indicate significance in two-tailed *t*-tests assuming unequal variance.

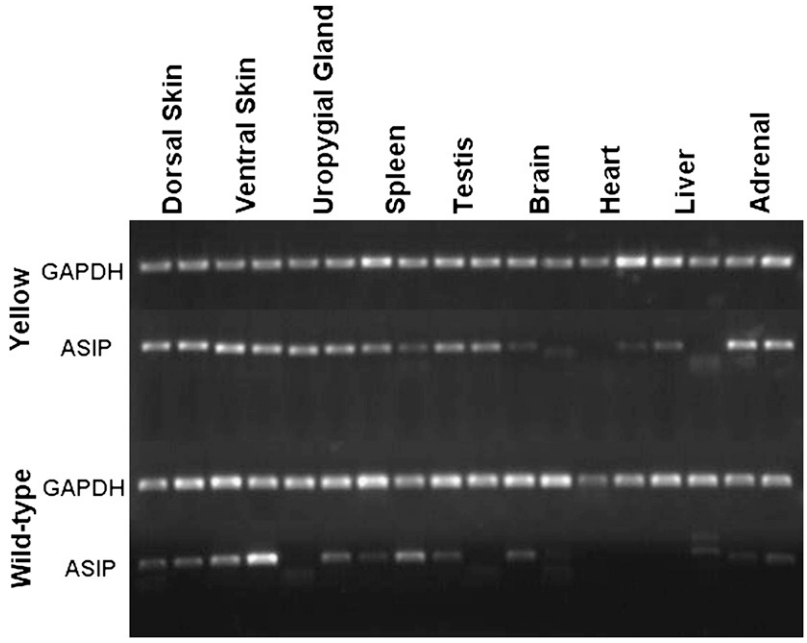


FIGURE 4.—RT–PCR analysis of ASIP mRNA expression in various tissues of adult wild-type (bottom) and dominant *yellow* (top) Japanese quail ($n = 2$ each). RT–PCR products for *ASIP* (primers ASIPF1 and ASIPR1) and *GAPDH* are shown for each sample.

and uropygial gland. However, expression was far from ubiquitous with the kidney and skeletal muscle still showing no expression (not shown), and the brain, heart, and liver showing inconsistent expression.

Microarray results: A comparison of gene expression in plucked dorsal skin of six yellow quails and six wild-type quails revealed three transcripts significantly downregulated in *yellow* individuals compared to wild type (FDR < 0.001). These included *SLC24A5* (mean fold change of expression in *yellow* relative to wild type $=$ 0.68), a gene previously implicated in eumelanin synthesis (LAMASON *et al.* 2005), and *EIF2B* (mean fold change in *yellow* $= 0.63$), consistent with its deletion from the *yellow* allele. The third transcript has homology to a gene involved in fatty acid metabolism and so may be linked to the abnormal fat metabolism in the *yellow* individuals. Twenty-five transcripts were significantly upregulated, including some candidates for lipid metabolism but no known pigmentation genes (supplemental Table 3 at http://www.genetics.org/supplemental/). Neither *ASIP* nor *RALY* was represented on the microarray. None of the Tyrosinase family genes (*TYR*, *TYRP1*, or *DCT*) were significantly up- or downregulated (mean fold changes $= 0.96$, 0.77, and 1.15, respectively, FDR $>$ 0.177), nor was the homolog of mouse *MCM6* (mean fold change $= 0.96$, FDR > 0.177), a gene identified as being upregulated by *ASIP* in murine melanocytes (FURUMURA *et al.* 1998), nor the homolog of *OCA2* (also known as *p protein*, mean fold change $= 1.17$, FDR $>$ 0.177), a gene downregulated by *ASIP* in mammals (RINCHIK *et al.* 1993). A homolog of *ITF2* (also called *TcF-4*), a transcription factor found to be upregulated by *ASIP* in mammalian melanocytes (FURUMURA *et al.* 1998, 2001), was represented on the array but failed to pass the filtering criteria. Significant downregulation of *SLC24A5* in *yellow* was confirmed by quantitative RT–PCR (mean fold change $= 0.38$ and 0.64, *t*-test $P = 0.010$ and 0.016 for normalization to β-*actin* and *GAPDH*, respectively).

***ASIP* in the chicken:** Our results confirmed the sequences of the three chicken 5′-noncoding exons. As in the quail, each was directly spliced onto exon 2, producing three different isoforms with 1b being largely ventral specific and 1c found in both dorsal and ventral skin (Figure 2). This confirms that 1b has been either extensively shortened in quails or lengthened in chickens. The expression of 1a in chicken differed from that in quail in being ventral specific. However, due to the short sequenced region of this exon we cannot confirm that these are homologous isoforms in quail and chicken. Semiquantitative RT–PCR also revealed 2.3 times higher ventral than dorsal expression of the *ASIP* coding regions (Table 3, $P = 0.002$, two-tailed *t*-test).

DISCUSSION

The various lines of evidence presented here strongly suggest that Japanese quail *yellow* is a mutation of the regulatory regions of the avian *ASIP* gene. This is therefore some of the first evidence (see also HIRAGAKI *et al.* 2008, accompanying article in this issue) for a functional *ASIP* gene in birds with a role in pigmentation. There are striking similarities to the mouse *lethal yellow* (A^y) mutation. The quail *Y* allele appears to be under the control of a different promoter from the wild-type *ASIP* alleles, which lies >200 kb upstream and is upstream of the *RALY* gene. The evidence suggests that, as in mouse A^y, this is the *RALY* promoter and that *ASIP* is controlled via this because of a deletion removing the *RALY* gene. This is consistent with the homozygous

TABLE 3

Expression of *ASIP* in dorsal and ventral skin in chicken

Sample	Dorsal	Ventral
463	0.39	1.57
456	0.80	1.37
388	0.58	1.55
374	0.56	0.95
382	0.58	1.48
567	0.19	0.34
637	0.13	0.23
8868	0.27	0.74
9190	0.05	0.12
450	0.64	1.09

Expression is based on band brightness, normalized to *GAPDH*.

lethality of the *Y* allele, which in mice has been considered to be due to the complete loss of the *Raly* gene (Michaud *et al.* 1994). We identified a second locus *EIF2B*, which is completely or nearly completely deleted from the *Y* allele and is also present between *ASIP* and *RALY* in the mouse genome. The product of this locus, subunit 2β of eukaryotic translation initiation factor 2, has an important role in protein synthesis (Suragani *et al.* 2005) and therefore the lethality of the homozygous *yellow* condition may involve loss of function of this gene rather than or in addition to *RALY* in both quail and mice. The similarity of these mutations in mice and quails raises the question of whether two independent deletion events occurring in the same region could be explained by chance or if there are conserved genomic features making this region prone to deletion.

Despite the strong similarity of the mouse and quail lethal *yellow* mutations at the genotypic level there appear to be some interesting differences in the downstream effects of this mutation. Unlike in A^y mice, we did not detect a significant difference in *ASIP* mRNA expression in the skin of Y/y^+ compared to y^+/y^+ quails, in spite of the clear effect of this mutation on color phenotype. There seem to be two possible explanations for this incongruity. The first is that the novel promoter of the *Y* allele does not increase the transcription of *ASIP* but instead produces mRNA that is more efficiently translated or has a post-translational effect that increases ASIP action.

The second explanation is that the *Y* allele does cause increased expression of *ASIP* but we were unable to detect this due to high variation in the background levels of *ASIP* expression in the skin samples in this study. This would be likely if *ASIP* was involved in producing within-feather banding or patterning in a similar way to the production of banded hairs in mammals. Expression would then be expected to vary across the feather growth cycle and be high when pale feather bands are being produced. This could explain the high variability in ventral/dorsal *ASIP* expression in wild-type individuals, as dorsal expression would be strongly influenced by the stage of feather growth, which was not controlled in these individuals.

This second explanation for the lack of a significant difference in *ASIP* expression between *yellow* and wild-type individuals is supported by *yellow*/wild-type expression being lower in plucked than in unplucked comparisons (Table 2), as this would be consistent with plucking causing upregulation of background levels of *ASIP*. Unlike the *yellow* mice, *yellow* quails are not homogenous in color but still exhibit feather and body region patterning albeit with restricted bands of eumelanin. If *ASIP* were responsible for these patterns in the wild-type birds, then the suggestion would be that the normal background expression is not completely masked by the mutant transcript, which again would be consistent with high variability and overlapping levels of expression between mutant and wild-type individuals.

Yellow quails also do not exhibit ubiquitous expression of *ASIP*, as is found in *yellow* mice (Bultman *et al.* 1992). There are two possible explanations for this, which we cannot currently differentiate: either the regulation of *RALY* is different between taxa with resulting differences in *ASIP* expression under the *RALY* promoter or *ASIP* expression in the quail, even when deregulated by this mutation, is kept in check by additional mechanisms not present in mice. Either way this difference may be the source of some of the phenotypic variation. For example, the lack of consistently increased *ASIP* expression in the brain of *yellow* quails could explain why no increase in food intake is observed (Minvielle *et al.* 2007), although more detailed studies on hypothalamic expression of *ASIP* will be required to confirm this.

Tissues in which we did find increased *ASIP* expression included the adrenal and uropygial (preen) glands. In mammals, antagonism of the MC2R in the adrenal gland causes decreased cortisol production. Therefore, increased *ASIP* expression in the adrenal gland could produce some of the differences in metabolism and increased lipogenesis observed in *yellow* quails (Minvielle *et al.* 2007). In chickens, MC3R, MC4R, and MC5R are all also expressed in the adrenal gland and show affinity for ACTH (Takeuchi and Takahashi 1998, 1999; Ling *et al.* 2004), so these could also be involved in mediating a response to *ASIP* within the adrenal gland. MC5R is expressed in chicken uropygial gland (Boswell and Takeuchi 2005) and in mammals the main function of this receptor is in regulating exocrine gland function. The fur of MC5R-deficient mice absorbs more water and takes longer to dry than that of wild-type mice due to reduced levels of hair lipids (Chen *et al.* 1997). Feathers plucked from around the uropygial gland of *yellow* quails showed a different response to wetting than did feathers from wild-type quails—water formed into droplets on wild-type but not on *yellow* feathers (F. Minvielle, un-

published data), suggesting that the role of MC5R on uropygial gland secretion merits further investigation.

While *ASIP* in both *yellow* and wild-type quail shows restricted expression, *AGRP* in chickens appears to have almost ubiquitous expression, being found in all tissues that have been examined, including all of those examined here (Takeuchi *et al.* 2000). The overlapping expression of these two inverse agonists must be regulated in different ways if both are active within the system. It seems likely that AGRP activity in different tissues could be regulated by differential affinity for the melanocortin receptor subtypes, as is found in mammals (Yang *et al.* 1999), while ASIP activity may be regulated via its differential expression patterns. However, this may not be the full story as we did find *ASIP* expression in wild-type quails in most tissues in which melanocortin receptors have been found, particularly those in which possible activity of these receptors has been documented, including brain, uropygial gland, spleen, and adrenal gland, albeit at low levels and not in all individuals.

We used the *yellow* quail to perform a preliminary assay for genes up- and downregulated in association with pheomelanogenesis on a chicken oligonucleotide microarray. Of most interest, the gene showing greatest downregulation in *yellow* quail was *SLC24A5*, and downregulation was confirmed by q-RT–PCR. *SLC24A5* encodes a melanosome membrane protein and has recently been shown to have a role in eumelanogenesis in humans and zebrafish (Lamason *et al.* 2005). Given the established link between this gene and pigmentation, its downregulation seems most likely to be due to effects of the *yellow* mutation on *ASIP* rather than on either of the other two genes (*RALY* or *EIF2B*) affected by the deletion. This suggests conservation of *SLC24A5* function in birds and, furthermore, that *SLC24A5* expression is negatively regulated by *ASIP*. In contrast, several genes did not show the predicted pattern from studies in mammals. In particular, we found no evidence for downregulation of the genes encoding the melanogenic enzymes of the Tyrosinase family (*TYR*, *TYRP1*, and *DCT*), as has been documented in response to ASIP in mammalian melanocytes (Kobayashi *et al.* 1995; Furumura *et al.* 1998, 2001; but see Voisey *et al.* 2003), although these are present in domestic fowl, with functions similar to those in mammals (Mochii *et al.* 1998; Chang *et al.* 2006; Nadeau *et al.* 2007b). One possible explanation for this is temporal variation in *ASIP* expression (see discussion above).

We have clearly demonstrated dorsoventral differences in expression of distinct *ASIP* mRNA isoforms in both wild-type quail and chicken, echoing the situation in mice. Further work will be required to explore the functional implications, including potential temporal-specific regulation of dorsal-expressed exons, but our results suggest that *ASIP* is involved in dorsoventral pigmentation patterning in both quails and chickens. The presence of an apparently functional *ASIP* gene in chickens poses the question of why no good candidates for mutations at this gene are known among the large numbers of described pigmentation variants (Smyth 1990). The finding of an expressed *ASIP* gene in birds, involved in dorsoventral pigmentation patterning, is interesting as it suggests a conserved basis for this type of pattern between birds and mammals. This backs up evidence suggesting that *ASIP* diverged from *AGRP* early in vertebrate evolution (Klovins and Schiöth 2005) and that *ASIP* may be involved in regulating dorsoventral pigmentation patterning in fish (Cerdá-Reverter *et al.* 2005). Fish differ from mammals and birds in that they have several different types of pigment cells (chromatophores); only one of these contains melanin (the melanophores) and only eumelanin, not pheomelanin (Ito and Wakamatsu 2003; Kelsh 2004). In fish, *ASIP* is expressed only in the ventral and not in the dorsal skin and seems to be involved in directing chromatophore differentiation, causing production of iridophores (structural pigment cells) and inhibiting production of melanophores. Therefore, it seems that the genetic pathways for bringing about pale ventral and dark dorsal pigmentation are ancient and conserved within vertebrates even though the cellular and biochemical basis of these patterns has changed, perhaps independently, in birds and mammals.

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