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The alpaca *agouti* gene: Genomic locus, transcripts and causative mutations of eumelanic and pheomelanic coat color

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ABSTRACT

The *agouti* gene encodes the agouti signaling protein (ASIP) which regulates pheomelanin and eumelanin synthesis in mammals. To investigate the role of *agouti* in coat color variation of alpaca, we characterized the *agouti* gene and identified three mutations potentially involved with the determinism of eumelanic and pheomelanic phenotypes. The exon-4 hosts the mutations g.3836C>T, g.3896G>A and g.3866_3923del57. Further analysis of these mutations revealed two genotypes for black animals. The reverse transcription analysis of mRNA purified from skin biopsies of alpaca revealed the presence of three transcripts with different 5′ untranslated regions (UTRs) and color specific expression. The white specific transcript, possibly originating from a duplication event (intra-chromosomal recombination) of the *agouti* gene is characterise by a 5′UTR containing 142 bp of the *NCOA6* gene sequence. Furthermore, the relative level expression analysis of mRNA demonstrates that the *agouti* gene has up-regulated expression in white skin, suggesting a pleiotropic effect of *agouti* in the white phenotype. Our findings refine the structure of the *agouti* locus and transcripts and provide additional information in order to understand the role of agouti in the pigmentation of alpaca.

1. Introduction

Pigmentation in mammals is known to be influenced by more than 350 genes (Montoliu et al., 2011). Among these, *agouti* is a well studied gene which encodes for a small secreted factor (agouti signaling protein) that normally functions as a paracrine regulator of hair pigmentation in mice and other mammals (Lamoreux et al., 2010; Vage et al., 1997; Wang et al., 1998). Mutually exclusive binding of the melanocortin-1-receptor (MC1R) by the agouti signaling protein and the α-melanocyte stimulating hormone (α-MSH) signals the hair-bulb melanocytes to synthesize pheomelanin (yellow–red pigments) and eumelanin (dark pigments), respectively (Jackson, 1994). In all the analyzed mammalian species the *agouti* transcripts are approximately 800 bp long and encode a protein of 131–133 amino acids. So far more than 50 *agouti* alleles have been identified in mice (Montoliu et al., 2011) and several studies have investigated the molecular genetics of *agouti* in other mammals and its association with pigmentation phenotypes. Recessive mutations at the *agouti* locus, which impair either agouti protein activity or alter the level of *agouti* mRNA synthesis, resulting in a darker coat color have been documented in many mammals, such as rodents (Kingsley et al., 2009; Linnen et al., 2009; Steiner et al., 2007), horses (Rieder et al., 2001), cats (Eizirik et al., 2003) foxes (Vage et al., 1997), and rabbits (Fontanesi et al., 2010). Moreover, other *agouti* alleles such as A^y (lethal yellow), which displays constitutive and ubiquitous expression of agouti, increases yellow pigmentation in mice (Miller et al., 1993). Apart from the yellow coat, mice that carry A^y display obesity, insulin resistance, premature infertility, increased body length and tumor susceptibility (Duhl et al., 1994).

In alpaca (*Vicugna pacos*) a wide variety of colors exists and it is therefore preferred in the fiber industry for the manufacturing of ecologically sustainable and organic textile products. The Peruvians always sought to protect the alpaca industry. Peru was the last South American country to open its borders to exportation and only approximately 3000 alpacas have actually left Peruvian soil. At the moment, Peru has four million alpacas representing approximately 90% of the global population. Therefore, this South-American country hosts the largest reserve of alpaca biological resources in the world. An estimated 20% of colored wool is presently produced in Peru and the alpaca fiber exportation represents one of the major economies for Peru, thus there is an increasing interest in better understanding the genetic basis of coat color in the Peruvian alpaca population.

So far, the main body of studies on alpaca has mainly regarded the analysis of the inheritance of phenotypes (Gandarillas, 1971; Valbonesi et al., 2009, 2011; Velasco et al., 1978a, 1978b), the biochemical properties of melanin (Cecchi et al, 2011; Fan et al., 2010; Renieri et al., 1991, 1995), and the morphology of melanosomes (Cozzali et al., 1998, 2001), and only few studies have focused on

Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; mRNA, messenger RNA; bp, base pair; kb, kilobase; nt, nucleotide; cDNA, complementary DNA; PCR, polymerase chain reaction; RT, reverse transcription; BLAST, Basic Local Alignment Search Tool; NCBI, National Center for Biotechnology Information.

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the molecular characterization of candidate genes responsible for coat colors, e.g. *MC1R* from American (Powell et al., 2008) and Australian (Feeley and Munyard, 2009) alpaca populations. A preliminary characterization of full length *agouti* transcripts from skin biopsies from an experimental trial for color segregation in Peruvian alpacas (Bathrachalam et al., 2012) and on the coding sequences of the same gene from genomic DNA isolated from blood samples from random Australian alpaca populations have recently been carried out (Feeley et al. 2011). Both studies have identified two missense mutations (g.3836C>T, g.3896G>A) and Feeley et al. (2011) also reported a 57 bp deletion (g.3866_3923del57), all located in exon 4 of *agouti* gene which codes for the last 40 amino acid residues constituting the Cys-rich C-terminal portion of ASIP, a domain of crucial importance for the functionality of the protein itself (McNulty et al., 2005). All these are probable loss-of-function mutations and they seem to be strongly associated with black color even if they neither explain all black phenotypes in alpaca nor all the phenotypic variation possible in ASIP (Feeley et al., 2011). Many other factors individually or in combination such as, mutations in regulatory region, genomic reorganization events (duplications and/or inversion), effects due to dilution genes (MATP, TYR, TYRP1) or contribution of MC1R genotypes (for black phenotypes) might be involved in the determination of the large variety of coat color observed in alpaca.

Therefore, the present investigations have been conducted to elucidate the genetic mechanisms behind alpaca pigmentation with a special emphasis on the characterization of the genomic and transcript structures, the relative levels of mRNA expression as well as, the description of mutations that probably affect eumelanin and pheomelanin synthesis from the skin biopsies of the native Peruvian alpaca population. These mutations have been analyzed in black, brown and white Peruvian alpacas involved in an experimental trial for color segregation analysis as described by Valbonesi et al. (2011).

2. Materials and methods

2.1. Collection and storage of skin biopsies

Skin biopsies from white and colored (brown and black) alpacas were collected by disposable biopsy punch (8 mm diameter) in RNAlater (SIGMA, Germany) from ILPA-Puno, Quimsachata Experimental Station, Instituto Nacional de Innovacion Agraria (INIA), Peru which is located at 4300 m above sea level. The alpacas analyzed in the present experiment were part of a previous phenotypic segregation study on coat color inheritance (Valbonesi et al., 2011). It should be noted that, due to the difficulties in sampling at this higher altitude, the total number of skin biopsies was limited to 80 samples. The biopsies were transferred to the School of Environmental Sciences, University of Camerino, Italy. Subsequently, they were removed from RNAlater, blotted with sterile blotting paper and stored at −196 °C (liquid nitrogen) for further analysis. All experiments were approved and performed according to the guidelines of the Animal Ethics Committee of the University of Camerino.

2.2. Nucleic acid extraction and cDNA synthesis

Total RNA from stored skin biopsies was extracted using an RNAeasy fibrous tissue mini kit (Qiagen S.A., Courtaboeuf, France). Simultaneously, genomic DNA was also isolated using a DNAeasy tissue kit (Qiagen S.A., Courtaboeuf, France) according to the manufacturer's instruction. The isolated RNA and DNA samples were stored at −80 °C for further analysis. The first strand cDNA was synthesized with 2 μg of total RNA using 10 pmol OdTm primer (Supplementary Table 1 and Supplementary Fig. S1), 0.5 mM dNTPs (Roche), 1× RT buffer, 20 U RNase inhibitor and 200 U PrimScript™ Reverse Transcriptase (Takara Biotech, Japan) in 20 μl total reaction volume according to the manufacturer's instructions. The reaction mixture was incubated for 45 min at 50 °C and then at 70 °C for 15 min and the resulting cDNA was used in coding sequence and 3′ end amplification. All the reverse transcription reactions were carried out using a Perkin-Elmer Thermal Cycler (Perkin-Elmer Corporation, Norwalk, CT, USA).

2.3. Primer design and PCR amplification of full length agouti transcripts by rapid amplification of cDNA ends (RACE)

Orthologous sequences of the *agouti* gene from mammals were retrieved from NCBI GenBank (http://www.ncbi.nlm.nih.gov/) and aligned with EMBL ClustalW (http://www.ebi.ac.uk//Tools/clustalw/) to identify conserved regions for the design of primers to amplify the coding region. PCR amplification of complete coding sequence from cDNA was done with the primers AF1/AR1 (Supplementary Table S1) in 50 μl reaction volume containing 1× Expand Long Range buffer (1.25 mM $MgCl_2$), 0.3 mM dNTP, 0.3 μmol of each primer, and 3.5 U Expand Long Range enzyme mix (Roche, Germany) with the following cycling condition: initial denaturation at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 66 °C for 30 s and 72 °C for 25 s, with a final extension at 72 °C for 7 min. The PCR products were cloned into the pGEM-T easy vector system (Promega, USA) and sequenced at BMR Genomics, Italy. The coding sequence was determined and used to design gene specific primers AF2, AR2, and AR3 (Supplementary Table S1 & Supplementary Fig. S1) for 3′ and 5′ RACE, a PCR approach specifically devised to characterize untranslated regulative region of the transcripts. The 3′ RACE was carried out with the primers AF2/nOdTm followed by amplification with AF1/OdTm primers (Supplementary Table S1 & Supplementary Fig. S1). Subsequently, 5′ RACE was carried out according to the method reported in Shi and Jarvis (2006). The first strand cDNA was synthesized with gene specific primer AR3, followed by amplification with UPML/AR1 primers and nested amplification was performed by using the UPMs/AR2 primer pair (Supplementary Table S1 & Supplementary Fig. S1). The amplifications were carried out as described above except for the annealing temperature (Supplementary Table S1) and the PCR products were cloned into the pGEM-T easy vector system and sequenced at STARseq, Germany. At least 5 independent clones were sequenced for each amplicon category.

2.4. Long range PCR

Expand Long-Template PCR (Roche Diagnostics, Switzerland) was performed to amplify the whole (ATG-TGA) alpaca *agouti* gene by using the AF1/AR1 primers (Supplementary Table S1) according to the manufacturer's instruction. Amplification of alpaca DNA with the *agouti* primers produced a 3945 bp fragment; the product was cloned into the InsTAclone PCR Cloning Kit (Fermentas GMBH, Germany) and sequenced at STARseq, Germany.

2.5. Sequence analysis

Nucleic acid and protein database searches were performed using BLAST at the NCBI server and visualized with Circoletto (Darzentas, 2010). The cDNA and DNA sequence data were analyzed using DNASTAR 5.0 software (Dayhoff et al., 1978). The alignment of the amino acid sequence of *agouti* proteins were performed using ClustalW (Thompson et al., 1994). The mRNA secondary structure predictions were performed with m-fold (Zuker, 2003).

2.6. Semi-quantitative RT-PCR

Expression of the *agouti* gene in white and colored skin (brown and black) biopsies were analyzed on cDNA obtained as described above using AF3/AR4 primers (Supplementary Table 1). PCR was carried out in a 25 μl of reaction volume containing 1× Dream Taq