Mitf splice variants in the skin of white merino sheep

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Introduction

The story of Microphthalmia-associated transcription factor (*MITF*) began in 1992, when it was described in a transgenic mice with an insertional mutation (Tachibana et al. (1992)). This established transgenic line showed peculiar phenotypes, i.e. white coat color and *microphthalmia*. *Mitf* (Hodgkinson et al. (1993); Hughes et al. (1993)) and its human counterpart *MITF* (Tachibana et al. (1994)) contain a basic helix-loop-helix-leucine zipper (bHLH-LZ) structure, which is required for DNA binding and dimerization. In almost all mammals investigated so far, *Mitf* consists of at least five isoforms with distinct N-termini, viz., *Mitf*-A, B, C, H and M (Amae et al. (1998)); Fuse et al. (1999); Udono et al. (2000)). Here we summarize for the first time, *Mitf* splice variants in the skin of white merino sheep.

Material and methods

Collection of skin biopsies. Skin biopsies were collected from the white merino sheep using disposable, sterile, biopsy punch (8 mm), treated with RNA *Later* (Sigma), finally stored in liquid Nitrogen. Samples were recorded according to the farm technicians.

RNA isolation, cDNA synthesis (RT-PCR), cloning and sequencing. RNA was extracted using TRI Reagent (Sigma) as per the manufactures instructions. cDNA was synthesized with PrimScript™ Reverse Transcriptase (Takara Biotech) followed by RT-PCR, 5'& 3' RACE (Version 2.0, Invitrogen; Xianzong Shi and Donald L. Jarvis. (2006)). Selected amplicons were gel purified (Nucleospin, Macherey-Nagel, Germany), cloned in the TA cloning system (TOPO, Invitrogen; pGEM, Promega; INSTA, Fermentas; StrataClone-UA, Stratagene) and sequenced by the commercial vendors (StarSEQ, Germany; BMR sequencing, Italy).

Bioinformatics. Nucleotide and protein sequences were retrieved from public databases (http://www.ncbi.nlm.nih.gov, http://www.ensembl.org). ClustalW (Thompson et al. (1994)) was used to align sequences. Specific and degenerate primers were synthesized using Primer3 (v. 0.4.0, http://frodo.wi.mit.edu/primer3/), CODEHOP (http://bioinformatics.weizmann.ac.il/blocks/codehop.html). The TARGETSCAN program (Release 5.1, http://www.targetscan.org/) was used to locate conserved *microRNA* target sequences in 3'UTR. The exons from human, mouse and dog were used to locate the exons in the other species through BLAST (Altschul et al. (1990)) and BLAT searches (http://genome.ucsc.edu) (Kent. (2002)).

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Results and discussion

Reverse Transcriptase (RT)-PCR analysis of *Mitf* mRNA from the white merino skin biopsies revealed two basic splice variants with (+) and without (-) an 18bp insert in the coding region (CDS) for the amino acids: ACIFPT (figure 1:A). The presence of the 6-aminoacid insert had a profound effect on DNA binding potential (Hemesath et al. (1994)) stabilizing the basic domain/DNA complex. Five ovine splice variants (figure 1:B), with distinct N-terminus sequences were determined by 5'RACE. Their N-termini sequence alignment is shown in figure 2. Three of them were found to be the counterparts of human *MITF/Mitf*-M, E and A with the identity of 97%, 96%, and 97% respectively. The other two transcripts were found to be 'novel' and named as '*Mitf truncated forms-1, 2*' (395, 222bp, figure 1:B). *Mitf* isoform multiplicity with differential expression patterns as well as functional diversity and redundancy was explained by Fuse et al. (1999). This extensive Alternative Splicing (AS) event, GC content, SNPs, Sequential Duplications (SD) and conservation with other mammals has been shown in figure 3, in comparison to human *MITF*. Usage of different promoters, giving rise to protein products with different N-termini has been demonstrated to be important for tissue specific expression and to affect the transcriptional activation potential (Takemoto et al. (2002); Udono et al. (2000)). Furthermore, three different *Mitf* 3'UTRs (625, 1083, 3167bp, figure 1:C) were characterized by 3'RACE. Further studies are required to understand the relevance of these alternatively spliced transcripts in regulating the gene expression linked to coat color (*microRNAs*, Zhu et al. (2010); Francois et al. (2010)). In addition, identification of interacting partners of each of the unique N-terminal domain could be of significance since they can help to modulate the function of *Mitf* isoforms. The present study contributes to a better understanding of the multiplicity of *Mitf* splice variants, while the functional importance of most of these splice forms still remain to be verified.

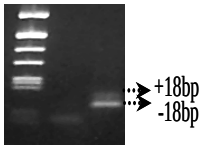

Figure 1:A

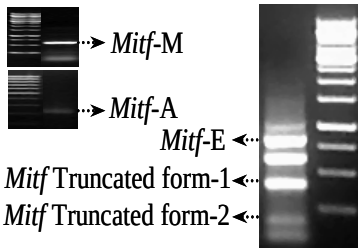

Figure 1:B

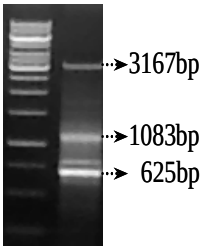

Figure 1:C

Figure 1: RT-PCR and RACE amplifications of the ovine *Mitf*. A) Ovine *Mitf* basic transcript variants (+/-18bp). B) 5'RACE amplification. Arrows indicate the position of the 5 identified *Mitf* 5'UTR amplicons. C) 3'RACE amplification. Arrows indicate the position of the 3 identified *Mitf* 3'UTR amplicons. DNA size markers: Gene ruler-100bp, 1kb and pBR322 DNA/AluI.

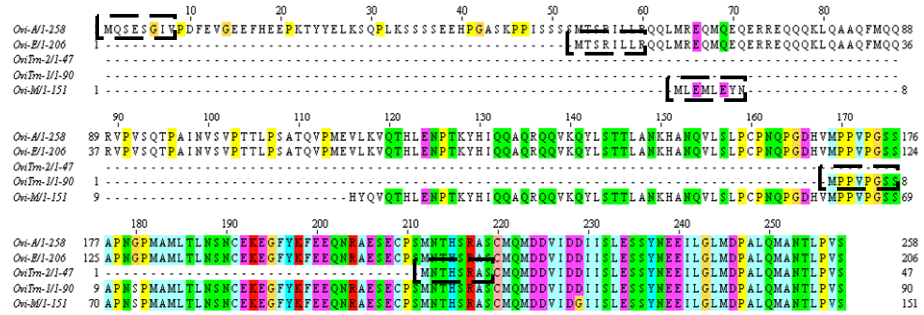

Figure 2

Figure 2: Ovine *Mitf* protein alignment. The rectangular boxes depict 5 different N-termini.

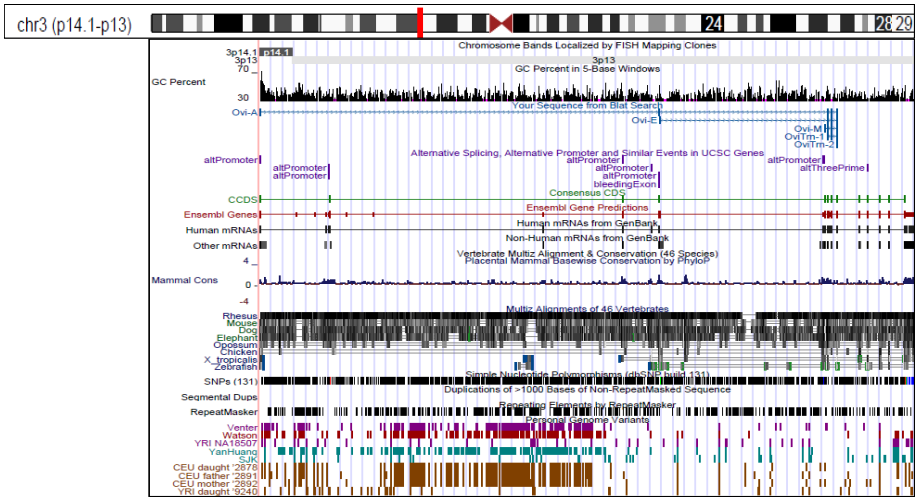

Figure 3

Figure 3: BLAT search of the ovine *Mitf* cDNA sequences with human *MITF* (chr. 3).

Conclusion

Mitf is an important stream flowing for pigmented cells. The widely spaced multiple promoters of the *Mitf* gene generates not only the diversity in the transcriptional regulation but also functionally different proteins. Further analysis of *Mitf* isoforms *in vivo* will elucidate its role in differentiation and development pathway of the pigmented cells.

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