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The mouse *silver* locus encodes a single transcript truncated by the *silver* mutation

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In mammals, melanin synthesis occurs mainly in highly differentiated cells, the epidermal melanocytes, where it is restricted to specialized organelles called melanosomes. Among the melanosomal proteins known to participate in the control of melanogenesis, those encoded at the silver locus (Kwon et al. 1991) are obviously important, since their impairment by the silver (*si*) mutation in mice results in premature graying of the hair owing to loss of follicular melanocytes (Kwon et al. 1991), but their function is disputed. It has been proposed that they are structural matrix proteins of the melanosome (Kobayashi et al. 1994). Other roles have been claimed, either as "stablins", proteins retarding melanogenesis by stabilization of biosynthetic intermediates, or as 5,6-dihydroxyindole-2-carboxylic acid polymerases (Chakraborty et al. 1996).

The homologous human SILV or PMEL17 gene (OMIM 155550, GDB 6277709) has been widely studied, since its protein products were proposed as potential markers of human melanoma and as immunotherapy targets. An apparently melanocyte-specific cDNA sequence was originally reported by Kwon et al. (1991; accession no. M77348, name PMEL17, also termed D12S53E). It was mapped to human Chromosome (Chr) 12pter-q21, sufficiently close to the murine silver locus (Chr 10; MGI 98301) to suggest similarity. The gene encodes a type 1 transmembrane protein of 668 amino acids, with a potential signal peptide, and putative membrane anchor domain near the C-terminus.

Soon thereafter, other cDNA clones were reported encoding a melanocyte-specific protein almost identical to PMEL17, termed either gp100 (as a 100-kDa glycoprotein; Adema et al. 1994), or ME20M (Maresh et al. 1994; accession no. M32295). These products were identical to each other. The amino acid differences from PMEL17 were one substitution (P274L) and the deletion of a heptapeptide (VPGILLT) located just before the transmembrane region (amino acids 588–594). Partial sequence analysis of genomic DNA indicated that gp100 and PMEL17 transcripts originated from a single gene via alternative splicing (Adema et al. 1994). This was confirmed once the full genomic organization and sequence of the human SILV locus were described (Bailin et al. 1996; Kim et al. 1996). The presence of the VPGILLT heptapeptide in PMEL17 but not in gp100 results from alternative mRNA splicing to two competing 3′ splice acceptor sites, whose function and regulation are unknown. Both proteins share all other identifiable domains, including the cytosolic ExxPLL motif proposed as a melanosomal targeting signal (Xu et al. 1997), as well as ten Ser, Pro, and Thr-rich tandem repeats in the melanosomal portion.

A murine *si*-derived cDNA named Pmel17 was also first cloned by Kwon et al. (1995; accession no. U14133). This sequence is called "Pmel17m" here for clarity. Comparison with the human sequences showed 77% nucleotide identity with PMEL17, but three main differences: (i) The central Ser/Thr/Pro-rich region is shorter in the murine protein; (ii) the predicted mouse "Pmel17m" protein lacks the heptapeptide distinguishing human PMEL17 from gp100, and so is actually a gp100 homolog; and (iii) there is a region of about 30 amino acids of low similarity to both human proteins in the C-terminal cytosolic domain.

Other cDNAs encoding murine homologs of gp100 have been reported and were called "gp100" (Schreurs et al. 1997; Zhai et al. 1997). These were identical and similar to Pmel17m except that the 3′ region of low similarity to the human sequences was missing. This was because in this region the murine gp100 sequence aligns numerically with the human, whereas there are three relative single-nucleotide deletions in Pmel17m, producing a local frameshift in the predicted protein compared with the human protein (Fig. 1, panel A). In the present work we will refer to this second murine gp100 homolog as "gp87" for clarity and in reference to the protein's lower molecular weight determined by SDS-PAGE followed by Western immunoblotting (Schreurs et al. 1997, and data not shown). The murine silver mutation was described as a single base insertion in this same region, causing a frameshift and extension of the protein by 12 amino acids (Kwon et al. 1995). The predicted mutant protein lacks the putative melanosomal targeting signal, suggesting misrouting of the protein. Interestingly, this insertion occurs exactly at the second position where Pmel17m has a deletion relative to gp87 (Fig. 1, panel A).

The reported differences between gp87 and Pmel17m are surprising and difficult to explain. It has not been shown whether these sequences can be expressed simultaneously or are allelic, nor whether any murine homolog of PMEL17, the longer human splicing variant, exists. This lack of knowledge of the exact nature and relationships of the murine silver products is a serious drawback for certain immunotherapy studies. We have, therefore, reexamined both the normal and silver mutant sequences, at the cDNA and genomic levels.

In order to detect murine silver transcripts, we first amplified cDNAs from B16-F10 melanoma cells, wild-type at the silver locus. The primers used (SF1/SR1) were common to the reported Pmel17m and gp87 transcripts and were chosen to amplify the

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The nucleotide sequence data reported in this paper have been submitted to Genbank and have been assigned the accession numbers AF118450 (wild sequence) and AF119092 (*silver* mutation).

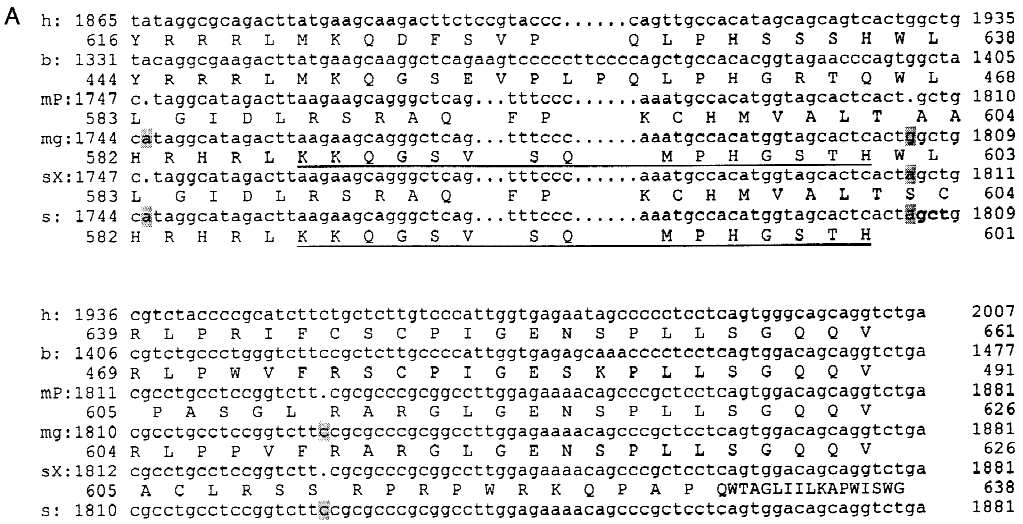

Fig. 1. Partial nucleotide and predicted protein sequences of the 3′ ends of *si*-related cDNAs, and genomic structure of the 3′ half of the gene. **Panel A.** Sequences of the C-terminal cytosolic domain. Numbers indicate the nucleotide or amino acid positions. h: human PMEL17 (Adema et al. 1994). This final portion is identical for the two human splice variants, but the numeration corresponds to the shorter form, gp100; b: bovine RPE (Kim and Wistow 1992); mP: murine Pmel17m (Kwon et al. 1995); mg: murine gp87 (Schreurs et al. 1997; Zhai et al. 1997; this paper); sX: silver mutant cDNA (Kwon et al. 1995), with the extended sequence compressed to fit the page, s: silver mutant cDNA (this paper). The peptide used to generate the antiserum αmg87 is underlined. The three differences between Pmel17m (Kwon et al. 1995) and gp87 are shadowed. Dots represent gaps for best alignment. **Panel B.** genomic structure of the 3′ half of the human and mouse silver genes. Introns are shown as lines and exons as boxes, with the number of the corresponding nucleotides in parentheses. The PCR primers used are shown by arrows. Their sequences are: Forward: SF1, GGT*ACTAGT*GCTGACCTCAGCGGTCA; SF2, CCCTCTGGAACCACAGTTGTACAA; SF3, GGT*ACTAGT*GTGTCTTTGGCTGACGC; SS, GTCCTTGCATCTCTGAT. Reverse: SR1, CAT*GAATTC*ACCATGTGGCATTTGGG; SR2, CAT*GAATTC*GTCAACCCCAGGAT. Primers SF1 and SF3 include an *Spe*I restriction site and both reverse primers *Eco*RI sites (italics), used for cloning. The position corresponding to the melanosomal transmembrane fragment (TM, exon 10), and the location of the peptide recognized by the αmgp87 antiserum (ep, exon 11) are shown as shadowed boxes. The wild type stop codon in exon 11 is marked by a star. The three single base differences between pmel17m and gp87 are indicated on top of the corresponding exons. The A substitution in the silver mutation is shown on top of the wild-type G, and the resulting stop codon highlighted with two stars. The sequence of the human and mouse intron 9 is shown in full: lowercase, intron 9; bold uppercase, flanking exons; italic uppercase, the 21-base segment found in only the longer human transcript. Best fits to branch sites in the human sequence (Adema et al. 1994) are marked B1, B2, and acceptor sites SA1, SA2. The A/G change in the mouse first branch site compared with the human is shown in shadowed uppercase.

region of cDNA corresponding to approximately the C-terminal half of the protein, where Pmel17m and gp87 are less homologous. Two independently cloned products yielded a sequence consistent with those published for mouse gp87 cDNA (Schreurs et al. 1997; Zhai et al. 1997) and thus displaying three-point insertions compared with Pmel17m (Fig. 1): A, G, and C at positions 1748, 1808, and 1830, respectively. These cause a frameshift in the predicted gp87 protein between codons 583 and 610, so that it more closely resembles human gp100 and PMEL17 and also the bovine homolog, retinal pigment epithelium (RPE)1 protein (Kim and Wistow 1992; Fig. 1, panel A), and the horse protein 17 precursor (accession no. AF076780). No other Pmel17 homologs are known in mammals. Like the published mouse gp87 sequences, neither of ours encoded a heptapeptide similar to that in human PMEL17.

Further attempts were made to clone a partial Pmel17m cDNA. RT-PCR was performed with two different forward primers (SF1 and SF2) and two reverse primers (SR1, located at the end of the coding region, and SR2, within the 3′ untranslated region of the gene), and two independent clones obtained with each set of primers were sequenced. However, both sequences corresponded to gp87. The repeated finding of gp87 but not Pmel17m sequences in B16 cells confirmed the expression of murine gp87 and indicated that, in these cells, Pmel17m transcripts are comparatively rare or absent. Moreover, the actual occurrence of murine gp87 was confirmed at the protein level. A polyclonal antiserum termed αmgp87 was raised against a synthetic peptide located in the cytosolic domain of the predicted gp87 protein, in a zone where the sequences of gp87 and Pmel17m are clearly different (underlined in

Fig. 1, panel A, and marked ep1 in panel B). This antiserum specifically recognized an 87-kDa protein in Western immunoblots performed with solubilized extracts from B16 cells (not shown).

Given the three-point differences between the Pmel17m and gp87 sequences, then if both transcripts actually occur, the genomic organization of the murine and human genes must be different. The two forms could conceivably arise from the alternative and mutually exclusive use of two highly homologous exons, one containing the three insertions. To test this and other possibilities, we examined the genomic organization of the 3′ end of the mouse silver locus. We used the SR2/SS and SR2/SF3 sets of primers to amplify, clone, and sequence overlapping fragments of the 3′ portion of the gene from B16 cells. The sequence obtained has been deposited in GenBank (accession no. AF118450). It contained two introns of 96 and 897 bp, an arrangement similar to that found in the human gene (Bailin et al. 1996). The exon sequence was identical to the gp87 cDNA sequence obtained by us and others. There was no alternative exon. Thus, the Pmel17m sequence is not encoded in B16-F10 cells. Interestingly, the genomic sequence of the putative intron 9 displayed a significant difference from the human sequence. In the human gene, intron 9 contains two potential splicing branch sites and two acceptor sites (Adema et al. 1994). This accounts for the occurrence of two species, PMEL17 and gp100, by alternative mRNA splicing to two competing 3′ splice acceptor sites. An essential A in the human 5′ branch site, necessary for formation of an intermediate lariat structure during splicing, is replaced in the mouse by a G, so that there is no functional branch site. Thus, the genomic structure of the mouse gene accounts for our inability to detect a cDNA sequence encoding an additional heptapeptide similar to that in human PMEL17. However, these results do not rule out the possible occurrence of splice variants involving the 5′ end of the gene.

Further possibilities concerning Pmel17m are that the three-point differences are errors or that they constitute a silver locus allelic variant different from that in B16 cells (used as cDNA source by us and both other groups who cloned murine gp87 sequences). The latter seemed unlikely, as the wild-type melanocytes used as source of the Pmel17m cDNA were from the same inbred strain of mice, C57BL/6J, as are B16 cells; however, inbred mice are not homozygous at all loci. The reported silver mutant sequence was similar to the Pmel17m sequence at two of the three positions in question (Kwon et al. 1995). Thus, we re-examined the silver (*si*) mutant sequence from the melan-si melanocyte line (Spanakis et al. 1992). There is only one *si* mutant allele, so this should be as reported (Kwon et al. 1995).

Two cDNA clones obtained from independent RT-PCR reactions with SF/SR2 primers gave the same sequence (GenBank accession no. AF119092). This matched the 3′-end of the *si* sequence given by Kwon et al. (1995) in having some neutral base changes compared with the (C57BL/6J) gp87 and Pmel17m sequences (Fig. 1, panel A), but differed from it by containing the first and third of the three insertions found in murine gp87. In the position of the second insertion (nt 1808), there was an A previously described as a pathological insertion (Kwon et al. 1995). However, if there is actually a G in this position of the wild-type sequence, as seen by all other groups, this A should be described as a substitution. This substitution generates a stop codon, truncating the predicted protein (Fig. 1). On the other hand, the neutral base differences seen by both us and Kwon et al. show that the normal mouse silver sequence is polymorphic, and the *si* mutation arose on a mouse strain background carrying an allele different from that in C57BL/6J mice.

In summary, we have addressed definitively the questions of the nucleotide sequence and number of transcripts arising from the 3′ end of the murine silver locus, and re-examined the pathogenic basis of the silver mutation. In human melanocytes, the SILV locus yields two transcripts differing by 21 bp, owing to alternative splicing to two 3′ acceptor sites in intron 9 (Adema et al. 1995; Bailin et al. 1996). Translation gives two proteins of 668 (PMEL17) and 661 (gp100) amino acids, expressed simultaneously in most human melanocytic cells (Adema et al. 1994), although gp100 mRNA is much more abundant than PMEL17 (Bailin et al. 1996). In mouse melanocytes, RT-PCR detected only mRNAs corresponding to the gp87 sequence. We propose the name gp87 according to the apparent molecular mass of the protein, until a definitive (perhaps functional) nomenclature is established.

We present several lines of evidence that gp87 mRNA is the only transcript from the *si* locus in the mouse. First, there is no Pmel17m variant transcript. After the initial description of this variant, only gp87 cDNAs were cloned from mouse cells by three groups including ours. Genomic sequencing shows that no such sequence was encoded at the silver locus. Second, and interestingly, there is no murine equivalent of the longer human splicing variant PMEL17. Again, sequencing of RT-PCR products from either murine source yielded no transcript containing the additional 21 bp insertion found in PMEL17. Again the genomic sequence was inconsistent with the production of such a splicing variant.

Sequencing of partial gp87 cDNAs from *si/si* mutant melanocytes showed that the *si* mutation consists of a G to A substitution at base 1808. Thus, rather than a frame-shift and extension, this substitution results in a premature stop codon and truncation of the protein in the C-terminal cytosolic domain (Fig. 1). The missing 25 amino acids include the putative melanosomal targeting motif ExxPLL (Xu et al. 1997). Thus, the pathogenic effect of the mutation is still attributable to misrouting of gp87 within the melanocyte, as suggested previously for the putative frame-shifted protein (Kwon et al. 1995).

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