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A melanocyte-specific gene, Pmel 17, maps near the silver coat color locus on mouse chromosome 10 and is in a syntenic region on human chromosome 12

(pigmentation/silver locus)

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ABSTRACT **Melanocytes preferentially express an mRNA species, Pmel 17, whose protein product cross-reacts with anti-tyrosinase antibodies and whose expression correlates with the melanin content. We have now analyzed the deduced protein structure and mapped its chromosomal location in mouse and human. The amino acid sequence deduced from the nucleotide sequence of the Pmel 17 cDNA showed that the protein is composed of 645 amino acids with a molecular weight of 68,600. The Pmel 17 protein contains a putative leader sequence and a potential membrane anchor segment, which indicates that this may be a membrane-associated protein in melanocytes. The deduced protein contains five potential N-glycosylation sites and relatively high levels of serine and threonine. Three repeats of a 26-amino acid motif appear in the middle of the molecule. The human Pmel 17 gene, designated *D12S53E*, maps to chromosome 12, region 12pter–q21; and the mouse homologue, designated *D12S53Eh*, maps to the distal region of mouse chromosome 10, a region also known to carry the coat color locus *si* (silver).**

A major obstacle in understanding the biology of pigmentation and pigment-related diseases is the lack of molecular probes specific for molecules involved in melanin biosynthesis. Tyrosinase catalyzes the first two steps of melanin biosynthesis (1–3) and, therefore, is a key enzyme. Tyrosinase-negative oculocutaneous albinism is due to inactivating mutations of the tyrosinase gene (4–6). Other albino mutants may, however, be positive for tyrosinase activity (7), which indicates that a block in melanin synthesis is not necessarily due to the absence of tyrosinase. Elucidation of the influence of additional factors in the control of melanization could be significant for the understanding of tyrosinase-positive albinism and variations in skin and hair pigmentation. For example, the silver (*si*) mutation induces hypopigmentation, which in the mouse involves the premature loss of many melanocytes from the hair bulb (8).

When we screened a λgt11 cDNA library of a primary culture of pigmented human melanocytes with anti-tyrosinase antibodies, 16 cDNA clones whose gene products reacted to anti-tyrosinase antibodies were isolated (9). Thirteen clones that cross-hybridized to each other were mapped to the mouse albino locus (*c*) and were characterized as the cDNA encoding human tyrosinase (9). The three remaining clones also cross-hybridized but did not share homology with the other group of clones. The representative cDNA clone was referred to as Pmel 17-1.†† We demonstrated that the gene for Pmel 17-1 did not map to the *c* locus on mouse chromosome 7 (10) and that the transcript of Pmel 17-1 was preferentially detected in melanocytes but not in nonpigmented cells.

Mouse molecular genetic linkage maps have proved invaluable for the structural and functional characterization of the mouse genome. They have been used to determine whether newly identified genes are homologous to known genes or classic mutations (11–15). In this study, we have analyzed interspecies crosses to map the mouse homologue of the Pmel 17 gene to chromosome 10 near *si* (silver), a mouse coat color locus.

MATERIALS AND METHODS

Isolation of cDNA Clones. Because the initial isolate of Pmel 17-1 was only 1.0 kilobase (kb), which was clearly not full-length, the human melanocyte cDNA library (9) was rescreened using a cDNA insert of clone Pmel 17-1 to obtain a longer cDNA. We isolated nine overlapping cDNA clones, and the nucleotide sequences of all of them were determined. The full-length cDNA of Pmel 17-1 is referred to as Pmel 17.

DNA Sequencing. Restriction fragments of DNA subcloned in M13 vectors were sequenced by the dideoxynucleotide chain-termination technique (16). A forward primer (New England Biolabs) complementary to the *lacZ* sequence adjacent to the 5′ side of the *Eco*RI site in λgt11 was used for direct sequencing of the end point of the cDNA insert in λgt11 (17).

Probes. Probes for Pmel 17-1 have been described (10). Probes for the *Gli*, *Ifg*, *Pah*, *Igf-1*, *pg*, and *Sl* loci have been described (11, 18–20).

Human–Hamster Somatic Cell Hybrids and Filter Hybridization. The primary chromosome assignment of Pmel 17-1 in the human was carried out with 16 hybrid clones that were derived from eight independent fusion experiments between Chinese hamster cell lines and human diploid fibroblasts or lymphocytes. The origin and characterization of these hybrids have been summarized (21, 22). For regional mapping of Pmel 17-1, we used a Chinese hamster–human hybrid clone with a spontaneous deletion of the distal long arm of human chromosome 12. The remaining region is 12pter–q21. Genomic DNA was extracted from cultured hybrid cells by routine

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procedures. Ten micrograms of hybrid and each parental control cell DNA was digested with a 4-fold excess of *Hin*dIII under conditions recommended by the manufacturer.

DNA fragments were separated by agarose gel electrophoresis and transferred to Hybond-N membranes (Amersham) by standard methods. After hybridization, the filters were washed and autoradiographed as described (23).

Mouse Gene Mapping and Filter Hybridization. Southern blot analysis was used to type the DNAs from a panel of Chinese hamster and mouse somatic cell hybrids (24) and from the progeny of the intersubspecies backcross [(NFS/N or C58/J × *Mus musculus musculus*)F_1 × *M. m. musculus*] (19), and the interspecies backcross [(C57BL/6J × *Mus spretus*)F_1 × C57BL/6J] (25). DNAs were digested by restriction endonucleases, electrophoresed on 0.4% agarose gels, transferred to nylon membranes, hybridized with ^{32}P-labeled probes, and washed as described (24, 26).

Recombination distances were calculated as described by Green (27) using the computer program SPRETUS MADNESS developed by D. Dave (Data Management Services, Frederick, MD) and A. M. Buchberg (National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD). Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

RESULTS

Sequence Analysis of Pmel 17. Fig. 1*A* shows the amino acid sequence deduced from the longest open reading frame of Pmel 17. The open reading frame encodes 668 amino acids with an M_r of 70,944. The first 23 amino acids show characteristics of a signal peptide of secretory or membrane-associated proteins and fit the -1, -3 rule (28) (Fig. 1). Inclusion of a histidine in a signal peptide is unusual but not unknown (28). Thus, the protein backbone of processed Pmel 17 would be composed of 645 amino acids with an M_r of 68,600.

Five potential asparagine-linked glycosylation sites are located at amino acid positions 81, 106, 111, 321, and 568 as indicated in Fig. 1 *A* and *C*. There is a stretch of 26 amino acids that constitutes a hydrophobic domain toward the C terminus of the protein (amino acids 598–623) (Fig. 1). This hydrophobic region is bordered by charged residues at either end (Glu^{597} and $Arg^{624-626}$), consistent with a transmembrane segment that makes a single helical span. One of the striking features of the protein is the relatively high percentage of serines (9.3%) and threonines (9.3%) and their peculiar arrangements. Although serine/threonine residues appear throughout the protein, we observed two sets of His-Ser-Ser-Ser (amino acids 202–205 and 639–642), one Gly-Ser-Ser (amino acids 302–304), four sets of Asp(Asn, Glu)-Thr/Ser-Thr/Ser (amino acids 264–266, 321–323, 370–372, and 425–427), six sets of Leu (Ile, Val, Ala)-Thr/Ser-Thr/Ser (amino

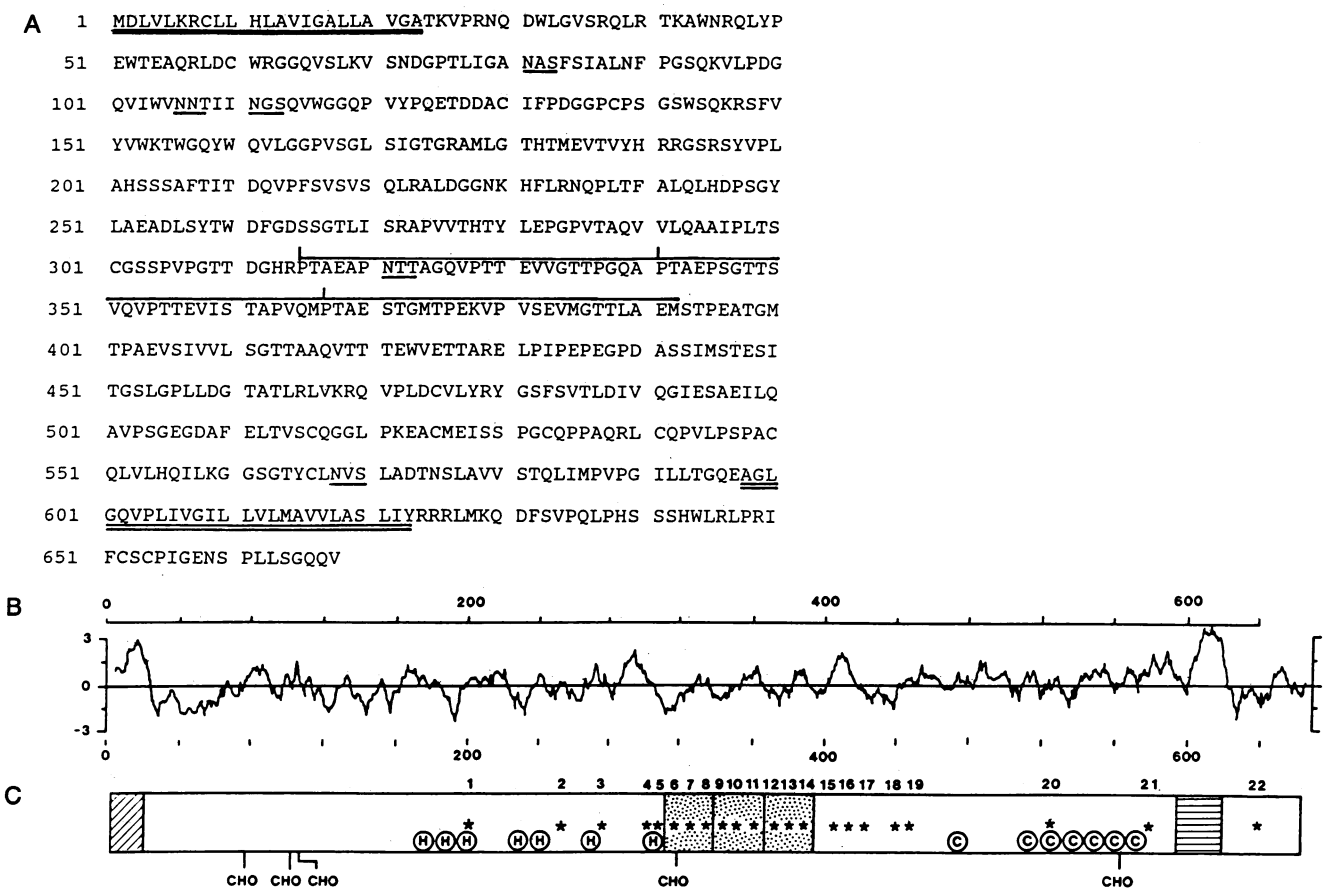

FIG. 1. Amino acid sequence analysis of the potential Pmel 17 protein. (*A*) Deduced amino acid sequence of Pmel 17. The signal region is underlined with a heavy line. The potential glycosylation sites are underlined with a thin line. The putative transmembrane region is doubly underlined. Three repeat motifs of 26 amino acids are indicated by an overline with vertical bars. Each bar indicates the start amino acid of the repeat. (*B*) Hydropathicity profile of the deduced amino acid sequence of Pmel 17. Local hydropathicity values calculated by the method of Kyte and Doolittle were plotted versus amino acid residues. Positive values indicate hydrophobic regions, and negative values indicate hydrophilic regions. (*C*) Schematic representation of the potential Pmel 17 protein. The entire coding region is boxed. The potential signal peptide is hatched. The transmembrane segment is indicated by horizontal lines. The central repeat motifs are stippled. Positions of histidines (H), cysteines (C), and possible N-linked glycosylation sites (CHO) are indicated. The numbers 1–22 on the box indicate the positions of asterisks, which indicate the positions of Ser/Thr-Ser/Thr sequences.

acids 298–300, 359–360, 418–420, 441–443, 528–530, and 580–582), five sets of Gly-Thr-Thr (amino acids 308–310, 334–336, 347–349, 386–388, and 412–414), two sets of Pro-Thr-Thr (amino acids 328–330 and 354–356), and two sets of Met-Ser-Thr (amino acids 392–394 and 445–447). The positions of the above two or three contiguous Ser/Thr-Ser/Thr sequences are indicated in Fig. 1*C* by asterisks with numbers. In addition, leucine, valine, and glycine are other amino acid residues present at high levels in this protein (11.9%, 10.0%, and 9.3%, respectively). There are three sets of 26-amino acid motifs in the middle of the molecule (amino acids 315–392) (Fig. 1 *A* and *C*). Each starts with four identical amino acids (Pro-Thr-Ala-Glu) and has a hydrophobicity pattern (Fig. 1*B*) similar to the others. The first two sets contain three Thr-Thr or Ser-Thr sequences. The third set contains one Ser-Thr and one Thr-Thr sequence. There is a histidine-rich region in front of the three 26-amino acid repeats and a cysteine-rich region following the repeat motif near the transmembrane domain (Fig. 1*C*). The significance of such an arrangement of amino acids in this protein remains to be determined. Finally, the relatively short C-terminal putative cytoplasmic domain contains a sequence Cys-Xaa-Cys-Pro (Fig. 1*A*), which is a binding site of protein tyrosine kinase $p56^{lck}$ (29). It will be interesting to determine whether Pmel 17 protein can bind to protein tyrosine kinases.

Human Gene Mapping. The human gene was mapped with a panel of Chinese hamster–human somatic cell hybrids, which contained different human chromosomes. After hybridization of the ^{32}P-labeled human Pmel 17-1 cDNA probe to *Hin*dIII-digested genomic DNA, a major 3-kb fragment was seen in human control DNA (Fig. 2, lane HSA), and a larger fragment in Chinese hamster control DNA (lane CH). In hybrids that contained human chromosome 12, a 3-kb band was observed in addition to the Chinese hamster band (lanes 4, 6, and 8). This band was absent in hybrids not containing human chromosome 12 (lanes 1–3, 5, 7, and 9). Thus, the presence of the 3-kb Pmel 17-1 sequence was completely concordant with the presence of human chromosome 12 in the 16 hybrid cell lines tested. All other chromosomes were excluded by at least two discordant hybrids. The number of discordant hybrids for each human chromosome is listed in parentheses after the chromosome number: 1(2), 2(3), 3(6), 4(4), 5(7), 6(3), 7(5), 8(5), 9(5), 10(5), 11(5), 12(0), 13(11), 14(8), 15(5), 16(4), 17(7), 18(6), 19(6), 20(6), 21(3), 22(6), X(2). These results assign the human gene for Pmel 17, termed *D12S53E*, to human chromosome 12.

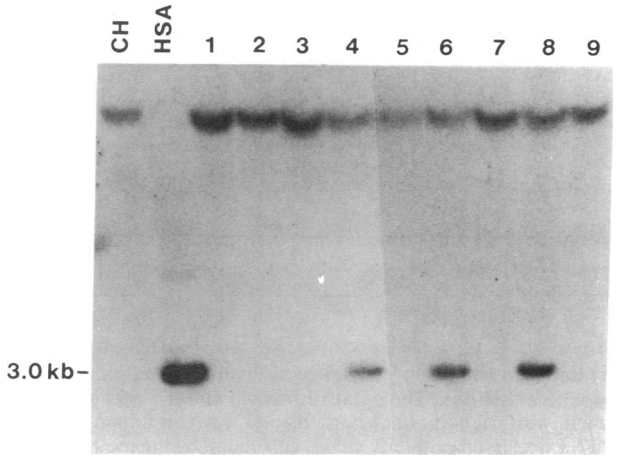

FIG. 2. Southern blot of *Hin*dIII-digested DNA of human (HSA) and Chinese hamster (CH) origin and from CH–HSA hybrid cell lines (lanes 1–9). Only the hybrids in lanes 4, 6, and 8 are positive for the human-specific 3-kb fragment.

In hopes of localizing this gene more precisely on chromosome 12, we used Southern blot analysis to examine a hybrid with a chromosome 12 deletion. The human 3-kb *Hin*dIII fragment was present in this hybrid. This hybrid retains only region 12pter–q21, placing the gene in this region. This hybrid is negative for the human *IGF1* locus at region 12q22–q24.1 (30).

Mouse Gene Mapping. The Pmel 17-1 clone was used as a hybridization probe to map the murine gene, designated *D12S53Eh*, in the mouse by analysis of a panel of 25 mouse–hamster somatic cell hybrids. Only two hybrids contained the 8.6-kb *Eco*RI fragment of the gene, and these were the only two hybrids in the panel that retained mouse chromosome 10 (data not shown). Since all other chromosomes were clearly discordant for Pmel 17-1, these data indicate that the murine counterpart of Pmel 17-1, termed *D12S53Eh*, is on chromosome 10.

To define a more precise location of *D12S53Eh* on mouse chromosome 10, we examined the progeny of an intersubspecies backcross and an interspecies backcross for restriction enzyme polymorphisms using Pmel 17-1 as a hybridization probe as well as other markers on chromosome 10 (11, 19). Pmel 17-1 identified an *Eco*RI fragment of 8.6 kb in the inbred strain parents of the cross between NFS/N and C58/J and 9.0 kb in the wild mouse-derived *M. m. musculus* (Skive) parent (Fig. 3). Thirty-eight of 90 backcross progeny inherited the inbred strain fragment, consistent with the expected 1:1 segregation ratio for a single gene. Comparison with the other markers previously mapped (19) on chromosome 10 indicates that the mouse homologue of this gene is at the distal end of chromosome 10 with gene order *lfg–Gli–D12S53Eh* (Table 1).

Analysis of interspecies backcross progeny derived from the cross [(C57BL/6J × *M. spretus*)F_1 × C57BL/6J] confirmed the chromosome 10 localization of Pmel 17-1. Pmel 17-1 detected 6.6- and 3.7-kb *Bgl* I fragments in C57BL/6J DNA and 14.0- and 2.4-kb *Bgl* I fragments in *M. spretus* DNA. The *M. spretus Bgl* I restriction fragment length polymorphisms were used to follow the segregation of the *D12S53Eh* locus in backcross mice. To position Pmel 17-1 on the linkage map, 167 backcross mice randomly selected from 205 progeny were typed for three additional loci. These three additional flanking loci were insulin-like growth factor 1 (*Igf-1*), steel (*Sl*), and pygmy (*pg*). The positions of these flanking loci on the interspecific linkage map have been determined (11). In addition to the 167 backcross animals typed for all six loci, up to 191 mice were typed for some pairs of loci. Each locus was analyzed in pairwise combinations for recombination frequencies using the additional data (20) as shown in Fig. 4.

The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are as follows: centromere–*Igf-1* (insulin-like growth factor 1)–*Sl* (steel)

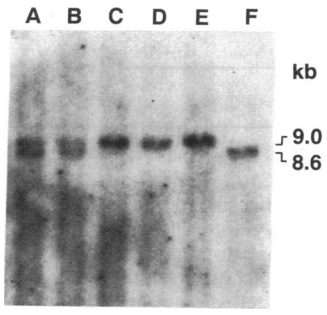

FIG. 3. Southern blot analysis of DNAs from the intersubspecies backcross using Pmel 17-1 as hybridization probe. Lanes A–E, individual backcross mice. Lane F, NFS/N.

Table 1. Segregation of the Pmel 17 hybridizing restriction fragment with alleles of *Ifg* and *Gli* in 90 progeny of an intersubspecies backcross

Mice	Inheritance of the NFS/N allele *Ifg*	*Gli*	*Mel*	No. of Mice
Parental	+	+	+	32
	−	−	−	51
Single recombinant	+	+	−	1
	−	−	+	2
	+	−	−	0
	−	+	+	4

Locus pairs	Recombination* r/n	% recombination ± SE
Ifg, Gli	4/90	4.4 ± 2.2
Gli, Mel 17	3/90	3.3 ± 1.9

*Percentage recombination between restriction fragments and SE were calculated according to Green (27) from the number of recombinants (*r*) in a sample size of *n*.

(29:184)–*Ifg* (interferon γ) (14:185)–*pg* (pygmy) (1:191)–*Gli* (glioblastoma oncogene) (4:188)–*D12S53Eh* (Pmel 17-1) (1:183). The recombination frequencies expressed as genetic distances in centimorgans ± SE are *Igf-1*–*Sl* (15.8 ± 2.7)–*Ifg* (7.6 ± 1.9)–*pg* (0.5 ± 0.5)–*Gli* (2.1 ± 1.1)–*D12S53Eh* (0.6 ± 0.5). There is a known locus distal to *Gli* that affects mouse coat color. The locus is called *si* or silver locus. The data indicate the possible localization of *D12S53Eh* near the *si* locus (31).

DISCUSSION

The overall makeup of the Pmel 17 protein is similar to tyrosinase and gp75 (32) encoded by the human *CAS2* gene (33) in that it has a signal sequence at the N terminus and a transmembrane domain toward the C terminus. As shown in Fig. 5, we also found amino acid similarity among the Pmel 17 protein (Pmel 17P), tyrosinase, and CAS2 protein (CAS2P, gp75). There are five regions of similarity in the three proteins: one in the N terminal portion, three in the middle, and one in the C terminus of the three molecules. Comparatively, the amino acid identity of the homologous regions is 30% between Pmel 17 protein and tyrosinase and 39% be-

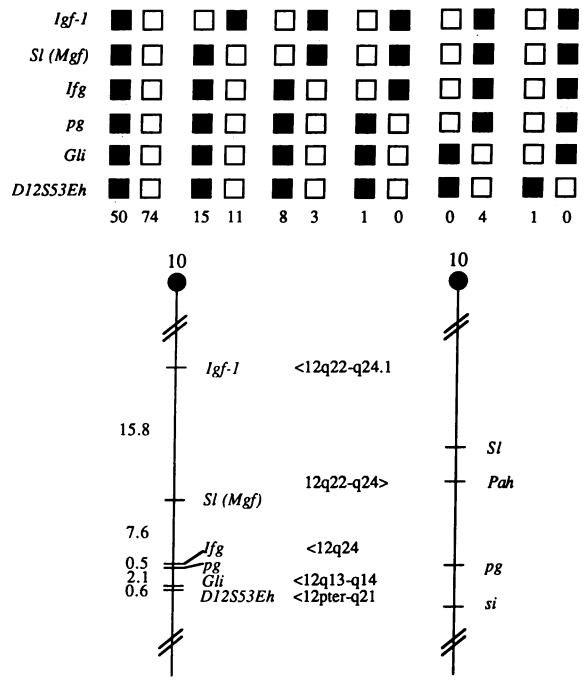

FIG. 4. Position of the *D12S53Eh* locus on mouse chromosome 10 from an analysis of an interspecies backcross. (*Upper*) Segregation patterns of Pmel 17-1 and flanking genes in 167 backcross animals are shown. For individual pairs of loci, more than 167 animals were typed with the probes shown. Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*)F_1 parent. Solid boxes, presence of a C57BL/6J allele; open boxes, presence of a *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. (*Lower Left*) A partial chromosome 10 linkage map showing the location of *D12S53Eh* in relation to linked genes in the interspecific backcross. Recombination distances between loci in centimorgans are shown to the left of the chromosome. (*Lower Right*) A partial chromosome 10 composite linkage map (from GBASE). The two maps were aligned at the *pg* locus. The position of loci mapped in humans is shown between the two maps.

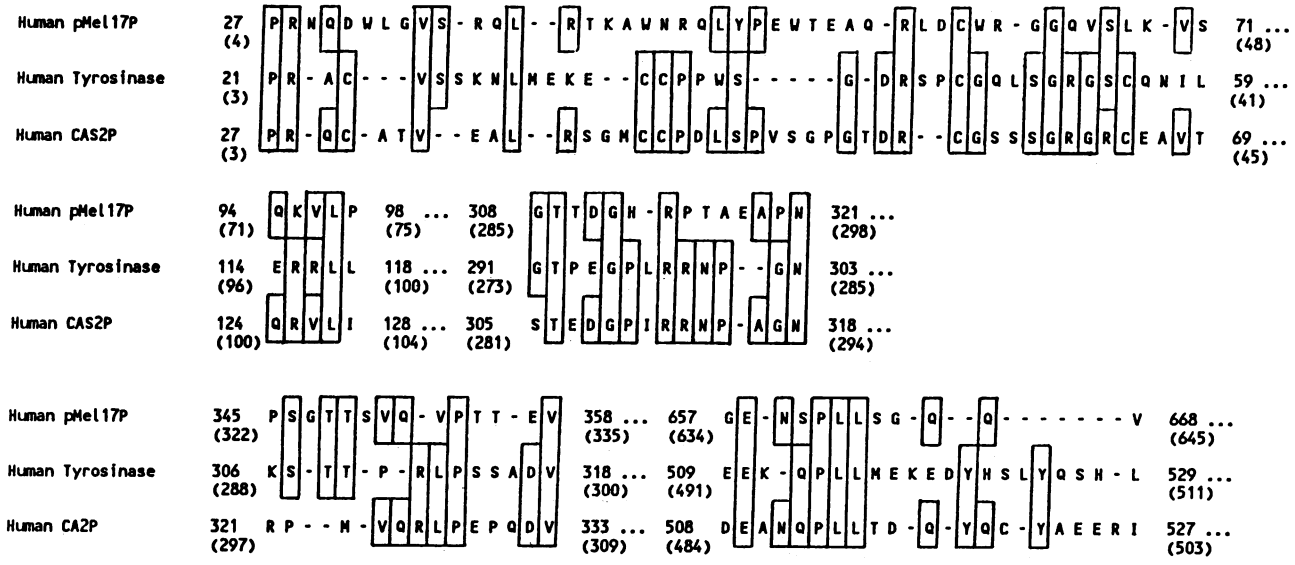

FIG. 5. Alignment of human Pmel 17 protein (Human pMel 17P), human tyrosinase, and human CAS2 protein (Human CAS2P) in homologous regions. Numbers indicate amino acid positions of the homologous regions, counting from the initiation residue, methionine, and those in parentheses indicate amino acid positions of potential mature proteins. The amino acids that are shared are boxed. Gaps (–) are introduced to allow maximal alignment.

tween Pmel 17 protein and CAS2 protein. Approximately 21% of amino acids in these regions are aligned in all three proteins. We also noted that three consecutive amino acids, Pro-Leu-Leu, are aligned in the C terminus of the three proteins (Fig. 5). If we take into account discrepancies between chemically similar amino acids, the similarity is 46% between Pmel 17 protein and tyrosinase, 54% between Pmel 17 protein and tyrosinase, and 54% between Pmel 17 protein and CAS2 protein. Direct sequence analysis with a λgt11 forward primer revealed that the open reading frame of Pmel 17-1 (a partial cDNA clone used for mapping) was in the frame with the *lacZ* gene of the λgt11 vector. This open reading frame of Pmel 17-1 spans from amino acid 141 to amino acid 435 of the Pmel 17 protein. The homology in the middle of the proteins might explain why polyclonal anti-tyrosinase antibodies recognized the Pmel 17-1 protein, and why the Pmel 17-1 protein competed with gp75 for one of anti-gp75 monoclonal antibodies in our earlier studies (10).

We do not know the significance of the peculiar appearance of the serines and threonines. It is possible that the serine/threonine residues might be sites for phosphorylation, which would be a potential means for the regulation of the activity of this protein, or they might be sites for O-glycosylation.

Previous studies (10) showed that Pmel 17 gene expression is more closely correlated with the level of melanin content than is tyrosinase expression. This suggests that Pmel 17 protein may function as a catalyst of melanin biosynthesis at a step distal to tyrosinase. It has been suggested that there are several steps at which enzymes other than tyrosinase could be involved; for example, dopachrome isomerase has been demonstrated to be involved in catalyzing dopachrome to 5,6-dihydroxyindole (34). The dopachrome isomerase is known to be inducible by β-melanotropin and isobutylmethylxanthine. The Pmel 17 mRNA is inducible by β-melanotropin and isobutylmethylxanthine. Such properties are similar to those of dopachrome isomerase.

Human chromosome 12 is known to contain a region of linkage homology (12p11–qter) to the distal region of mouse chromosome 10 (35). The human homologues of the mouse markers used to position *D12S53Eh* in the mouse *PAH*, *IGF-1*, *IFG*, and *GLI* are conserved in a syntenic group on human chromosome 12q. In humans, *PAH* maps to 12q22–q24, *IGF1* maps to 12q22–q24.1, *IFG* maps to 12q24, and *GLI* maps to 12q13–q14 (30, 36–38). The assignment of *D12S53Eh* distal to these markers extends this region of known homology on mouse chromosome 10.

In Fig. 4, we have aligned our interspecific linkage map with the composite linkage map (provided from GBASE, a computerized data base maintained at The Jackson Laboratory) using the *pg* locus as an anchor. This alignment places *D12S53Eh* near the *si* (silver) coat color locus that has been mapped to the distal region of chromosome 10 by conventional intraspecific crosses (Fig. 4). Nonagouti hairs of homozygous silver mice may be all white, all black, black with white tips, or white with gray or black bands. Silvering results from a reduction in the number of pigment granules (39). In histological studies, Quevedo *et al.* (40) observed that silvering resulted from a premature loss of melanocytes within the hair follicles. The cause of premature dislodgement of follicular melanocytes is unknown. The phenotype of *si* would be consistent with a defect in a melanocyte-specific gene such as *D12S53Eh*. Whether the *si* locus encodes the mouse homologue of Pmel 17 and, therefore, the mouse Pmel 17 protein represents the si protein remains to be determined.

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