# A Novel Splice Variant of Pmel17 Expressed by Human Melanocytes and Melanoma Cells Lacking Some of the Internal Repeats

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**Pmel17 is a ≈100 kDa pigment cell specific glycoprotein that plays a crucial part in the morphogenesis of melanosome precursors. Anti-Pmel17 immunoprecipitates from metabolically pulse labeled melanoma cells and melanocytes contain, in addition to full-length Pmel17, a glycoprotein that migrates with a lower relative molecular weight. Here we show that this glycoprotein is encoded by an mRNA that results from alternative splicing of the human Pmel17 gene from which a cryptic intron is excised. Immunoprecipitation recapture experiments showed that this glycoprotein contained both the N- and C-termini of full-length Pmel17. Sequence analysis of cDNA corresponding to the alternatively spliced form reveals the loss of three of 10 imperfect direct repeats from the central region of the lumenal domain. The product of the splice variant is processed with similar kinetics to full-length Pmel17, and localizes similarly to late endosomes when expressed ectopically in nonpigment cells. We speculate that truncation of the repeat region within Pmel17 alters either fibrillogenic activity or the interaction of Pmel17 with melanin intermediates. The expression of an alternatively spliced product may furthermore affect the cohort of peptides generated for recognition of melanoma cells by tumor-directed T lymphocytes.** ***Key words: fibril formation/gp100/melanoma/melanosome/silver. J Invest Dermatol 121:821–830, 2003***

Melanin biosynthesis and storage are sequestered within lysosome-related organelles of melanocytes and eye pigment epithelia called melanosomes (Orlow, 1995; Marks and Seabra, 2001). In order to carry out their unique function, melanosomes harbor a cohort of glycoproteins that are expressed only in pigment cells. These proteins include enzymes, such as tyrosinase and the tyrosinase-related proteins Tyrp1 and Dopachrome tautomerase, as well as structural proteins and transporters that modulate the morphology, intralumenal microenvironment, and function of the melanosome (Sturm *et al*, 2001). Melanosome activity and morphology and the type of melanins produced can be modulated by the expression or modification of various components through gene regulatory mechanisms or mutation (Hearing, 1999, 2000). This kind of modulation can affect pigmentation in primary melanocytes or tumorigenicity and/or immunogenicity in transformed melanocytes (Kawakami *et al*, 2000; Overwijk and Restifo, 2000; Slingluff *et al*, 2000).

Pmel17 (also known as gp100 or the product of the *Silver* locus) is a type I integral membrane glycoprotein that localizes to the lumen of melanosome precursors (Kwon *et al*, 1987; Orlow *et al*, 1993; Kobayashi *et al*, 1994; Lee *et al*, 1996). It is highly expressed by melanocytes, and serves as a common target for tumor-directed T lymphocytes in patients with melanoma (Kawakami *et al*, 1998a). We have shown that Pmel17 aligns with the intralumenal fibers that are characteristic of melanosomes and their precursors (Berson *et al*, 2001; Raposo *et al*, 2001), that ectopic expression of Pmel17 alone is sufficient to induce the formation of similar fibers in nonpigment cells (Berson *et al*, 2001), and that a lumenal domain fragment copurifies with the fibers (Berson *et al*, 2003). Others have implicated roles for Pmel17 in immobilizing the melanin intermediate, DHICA (Chakraborty *et al*, 1996; Lee *et al*, 1996), and in detoxifying DHICA and/or other melanin intermediates within the melanocyte (Quevedo *et al*, 1981; Spanakis *et al*, 1992); both of these activities may be associated with the fibrillogenic activity of Pmel17, resulting in immobilization of melanin intermediates on fibrous structures. The hypopigmentation of *silver* mice, which harbor a truncated Pmel17 molecule (Martínez-Esparza *et al*, 1999), indicates that an intact cytoplasmic domain is required for Pmel17 function. The structural features of the Pmel17 lumenal domain that effect function within melanosomes, however, are not known. During its biosynthesis within melanocytes, Pmel17 undergoes complex processing, involving cleavage within the lumenal domain, which is required for its fibrillogenic activity (Berson *et al*, 2001, 2003), suggesting that its function may require conformational changes induced by proteolytic processing of this domain. The only other recognizable features of the lumenal domain are (1) a small region with homology to polycystic kidney disease (PKD) repeat regions within the PKD 1 protein (Hughes *et al*, 1995), and (2) 10 tandem repeats of a 13 amino acid proline- and glutamate-rich sequence immediately downstream of the PKD repeats (Kwon *et al*, 1991).

Manuscript received February 9, 2003; revised March 20, 2003; accepted for publication April 20, 2003

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Abbreviations: EndoH, endoglycosidase H.

0022-202X/03/$15.00 · 

Two distinct Pmel17 products resulting from alternative splicing of the mRNA have been described in human melanocytic cells (Adema *et al*, 1994; Maresh *et al*, 1994; Bailin *et al*, 1996; Kim *et al*, 1996). These two forms differ by only seven amino acids, encoded by a 21 bp insertion resulting from use of an alternative splice acceptor site in exon 10 of the Pmel17 gene, within the juxtamembrane region of the lumenal domain (Bailin *et al*, 1996; Kim *et al*, 1996). Here, we describe a second splice variation that occurs in human melanocytic cells and results in the production of a smaller Pmel17 product containing a deletion within the lumenal domain tandem repeat region. We speculate that expression of this truncated Pmel17 may alter oligomerization or melanin binding properties and thereby modulate melanosome structure and/or function.

## MATERIALS AND METHODS

**Cell culture and transfection** Human melanoma cell lines MNT-1 and 1011-mel were cultured in Dulbecco minimal Eagle's medium supplemented with 10% AIM-V medium, 20% fetal bovine serum, 1 mM sodium pyruvate, nonessential amino acids, and antibiotics (penicillin and streptomycin). Melan-a (Bennett *et al*, 1989) cells and the mouse T cell hybridoma, DO11.10 (Roehm *et al*, 1982) were cultured as described. Primary melanocytes, a kind gift from Dr M. Herlyn and R. Finko, were cultured as described (Raposo *et al*, 2001). HeLa cells were cultured as described (Calvo *et al*, 1999), and transfected using FuGene-6 (Roche Applied Science, Indianapolis, Indiana) according to the manufacturer's instructions with 2 μg of total plasmid DNA (0.2 μg of specific plasmid DNA and 1.8 μg of empty pCI expression vector).

**Antibodies** αPmel-N and αPmel-I antibodies were raised against peptides corresponding to N-terminal or internal regions of the Pmel17 lumenal domain. Peptides were synthesized with the following sequences, representing the indicated residues from full-length Pmel17 with an additional N-terminal cysteine added for coupling to keyhole limpet hemocyanin using m-maleimidobenzoyl-*N*-hydroxysuccinimide ester: Pmel-N, $H_2N$-(C)TKVPRNQDWLGVSRQLR-$CO_2H$ (residues 24–40); Pmel-I, $H_2N$-(C)QVPTTEVVGTTPGQAPTAE-$CO_2H$ (residues 326–344). Peptide synthesis, coupling, rabbit immunization and production were performed by Genemed Synthesis (South San Francisco, California). Anti-peptide antibodies were affinity purified by passing sodium sulfate precipitated serum through a column in which peptides were coupled to SulfoLink gel (Pierce Biotechnology, Rockford, Illinois) according to the manufacturer's instructions. Antibodies were eluted with 0.1 M glycine pH 2.7, neutralized with Tris base, and dialyzed in phosphate-buffered saline containing 0.02% sodium azide. Other antibodies and their sources were as follows: αPEP13h (referred to here as αPmel-C for simplification), to Pmel17 cytoplasmic domain (Berson *et al*, 2001); HMB50 and HMB45, to Pmel17 lumenal domain (Lab Vision, Fremont, California); and H4A3, to LAMP1 (Developmental Studies Hybridoma Bank, Iowa City, Iowa). Chromophore-conjugated secondary antibodies were obtained from Jackson Immunoresearch (West Grove, Pennsylvania) or from Southern Biotech (Birmingham, Alabama).

**Reverse transcriptase–polymerase chain reaction (reverse transcriptase–PCR) and cloning of alternative splice forms** mRNA was isolated from MNT-1, 1011-mel, HeLa, melan-a, cultured primary human melanocytes, or D011.10 mouse T cell hybridoma cells using the RNEasy kit (Qiagen, Valencia, California). For reverse transcriptase reactions, RNA (15 μg) was incubated with reverse primers, as indicated, heated to 90°C, cooled to 52°C for 15 min, and then incubated with Avian Myeloblastosis Virus reverse transcriptase and appropriate buffers according to the manufacturer's instructions (Promega, Madison, Wisconsin). Control reactions lacked reverse transcriptase. After ethanol precipitation, 12.5% of each reaction was subjected to PCR amplification with indicated primers for 30 cycles (1 min at 95°C, 1 min at 55°C, and 2.5 min at 72°C). Parallel reactions were done using 100 ng of plasmid DNA with Pmel17-l insert. Reaction products were fractionated by agarose gel electrophoresis alongside 100 bp markers (Life Technologies, Rockville, Maryland) and visualized after staining with ethidium bromide. Triads of PCR reactions with template from reverse transcriptase reactions that contained or lacked reverse transcriptase enzyme or with plasmid template are shown at identical exposures for each triad. Primers are indicated in **Table I**.

For sequencing and subcloning, PCR reaction products using primers 144 and 398 were fractionated by agarose gel electrophoresis, purified, and subcloned into pCR2.1 by TA cloning (Invitrogen, Carlsbad, California). Clones with appropriate sized *Eco*RI inserts for Pmel17-s and Pmel17-i were sequenced by automated dideoxy sequence analysis (Children's Hospital, Philadelphia, Pennsylvania). To reconstitute a full-length cDNA for hPmel17-s, the *Nco*I–*Xba*I fragment from pCR2.1-hPmel17-s was used to replace the corresponding fragment in full-length hPmel17-l subcloned in pSP72 (Promega). Similarly, full-length hPmel17-i was generated by using the *Bst*XI–*Xba*I fragment from pCR2.1-hPmel17-i to replace the corresponding fragment in pSP72-hPmel17-l. From the resultant pSP72 plasmids containing full-length products, *Eco*RI–*Xba*I fragments containing the entire reading frame of hPmel17-s or hPmel17-i were subcloned into the pCI mammalian expression vector (Promega). Sequences of all inserts were verified by automated dideoxy sequencing.

**Metabolic pulse chase and immunoprecipitation analyses** Melanoma cells, melanocytes, or transfected HeLa cells were metabolically labeled with $^{35}$S-methionine/cysteine as described (Berson *et al*, 2000) for 20 to 30 min, then chased for 0 to 4 h as indicated. Cell lysates in 1% Triton X-100 were prepared and immunoprecipitated as described (Berson *et al*, 2000). For immunoprecipitation/recapture experiments, immunoprecipitated products were released by addition of 50 μL of 0.5% sodium dodecyl sulfate (SDS) and heating to 100°C for 5 min. Samples were then brought to 0.5 mL with lysis buffer to dilute the SDS, and subjected to a second round of immunoprecipitation with indicated antibodies. All immunoprecipitates were fractionated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and analyzed on a Molecular Dynamics Storm Phosphor Imaging system using ImageQuant software (Amersham Biosciences, Piscataway, New Jersey).

**Table I. Oligonucleotides used for reverse transcription–PCR**

| No. | Derivation | Position in cDNA[a] | Sequence of oligonucleotide[b] |
|---|---|---|---|
| 144 | hPmel17 | 451–474 | $^{457}$GCA TCT TCC CTG ATG GTG$^{474}$ |
| 145 | hPmel17 | 855–872 | $^{855}$GGA GAC AGT AGT GGA ACC$^{872}$ |
| 147 | hPmel17 | 1654–1670 | $^{1654}$CAT CGC CAG GGT GCC AG$^{1670}$ |
| 166 | hPmel17 | 1938–1952 | *GGG CTC ACC TGG CAA* $^{1938}$AGG CGC AGA CTT ATG$^{1952}$ |
| 171 | hPmel17 | 1454–1492 | $^{1454}$C ACC TTA AGG CTG GTG *CAG CAA* CAA GTC CCC CTG GAT TG$^{1492}$ |
| 172 | hPmel17 | 1492–1454 (R) | $^{1492}$CA ATC CAG GGG GAC TTG *TTG CTG* CAC CAG CCT TAA GGT G$^{1454}$ |
| 356 | hPmel17 | 1998–1983 (R) | *tc ttt tct aga tt*$^{1998}$A GTG ACT GCT GCT ATG$^{1983}$ |
| 398 | hPmel17 | 2119–2102 (R) | *tgt ttt cta ga*$^{2119}$g ttt ctg tca act cca gg$^{2102}$ |
| 232 | mGAPDH | 47–69 | $^{47}$ATG GTG AAG GTC GGT GTG AAC GG$^{69}$ |
| 233 | mGAPDH | 1045–1018 (R) | $^{1045}$CTC CTT GGA GGC CAT GTA GGC CAT *C*AG G$^{1018}$ |
| 319 | hRab5a | 57–73 | *atac cag atc ttg* $^{57}$ATG GCT AGT CGA GGC GC$^{73}$ |
| 321 | hRab5a | 704–682 (R) | *aaga tct aga* $^{704}$TTA GTT ACT ACA ACA CTG ATT CC$^{682}$ |
| 418 | mPmel17 | 902–921 | $^{902}$GT GGT TCC TCC CCA GTC CCG$^{921}$ |
| 419 | mPmel17 | 1320–1302 (R) | $^{1320}$CAG GGG AAC TTG TCT CTT C$^{1302}$ |

[a]Reverse primers are indicated by (R).
[b]Superfluous sequences that are not directly homologous to the cDNA are indicated by *italics*.

**Western blotting analyses** Whole cell lysates of transfected HeLa cells were treated or not with endoglycosidase H (EndoH, New England Biolabs, Beverly, MA), fractionated by SDS–PAGE, transferred to reinforced nitrocellulose membranes, probed with αPmel-N or αPmel-C antibodies, and developed with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin and ECF enhanced chemiluminescence (Amersham Biosciences) as described previously (Berson *et al*, 2001).

**Immunofluorescence microscopy analyses** Transiently transfected HeLa cells expressing hPmel17-l, hPmel17-i, or hPmel17-s were fixed with 2% formaldehyde in phosphate-buffered saline and stained as described (Calvo *et al*, 1999) with antibodies to Pmel17 (HMB50; IgG2a) and to Lamp1 (H4A3; IgG1), followed by isotype-specific secondary antibodies conjugated to fluorescein isothiocyanate or Texas Red. Cells were analyzed on a Leica Microsystems (Bannockburn, Illinois) DM IRBE microscope, and images were captured, analyzed, and processed for deconvolution using a Hamamatsu (Hamamatsu, Japan) Orca digital camera and Improvision (Lexington, Massachusetts) OpenLab software.

**Sequence analyses** Sequence formatting and comparisons were done using DNAStar (Madison, Wisconsin) or DNA Strider. The following GenBank accession nos were used to obtain cDNA sequences for Pmel17 orthologs from the NCBI database: mouse (*Mus musculus*) Silver, accession no. NM.021882; chicken (*Gallus gallus*) MMP115, accession no. D88348; bovine (*Bos taurus*) RPE1, accession no. M81193; and horse (*Equus caballus*) Pmel17, accession no. AF076780.

## RESULTS

**Band X is expressed in normal melanocytes in addition to melanoma cells** Anti-Pmel17 immunoprecipitates of cell lysates from metabolically pulse labeled MNT-1 human melanoma cells contained, in addition to the ≈100 kDa band representing full-length Pmel17, a band with faster migration (≈95 kDa; Berson *et al*, 2001). This band, referred to as "band X" was: (1) precipitable with either of two distinct antibodies to Pmel17 (HMB50, which recognizes the lumenal domain, and αPmel-C, which binds to the cytoplasmic domain), (2) susceptible to digestion with EndoH (indicating residence within a pre-Golgi compartment), and (3) not obvious in lysates from cells that were chased in the presence of excess unlabeled methionine (Berson *et al*, 2001; see also Berson *et al*, 2003). The band was distinguished from the proteolytic product, Mα, by virtue of its slightly distinct migration in gels and by its susceptibility to digestion with EndoH; Mα, which appears only after the chase, is resistant to EndoH digestion. Furthermore, band X was not observed in lysates of transfected HeLa cells expressing Pmel17 from a transgene (Berson *et al*, 2001).

To determine whether band X was unique to MNT-1 cells or represented a coprecipitating product present in untransformed cells, we subjected cultures of normal primary human melanocytes to a similar metabolic pulse/chase analysis. As shown in **Fig 1**, a similar pattern of immunoprecipitable products was observed from Triton X-100 cell lysates of these cells as previously observed in MNT-1 cells. Thus, band X was present in addition to full-length, core glycosylated "P1" Pmel17 at the pulse. During the chase, both were reduced in intensity and a slightly faster migrating Mα band appeared concomitantly with the ≈28 kDa Mβ and the faint, diffuse slower migrating P2 form representing the Golgi modified full-length protein (note that unlike P1 and band X, the migration of Mα and P2 varies among melanoma cells and primary melanocytes most likely due to differential terminal glycosylation; Vogel and Esclamado, 1988; Chiamenti *et al*, 1996). Although reduced in intensity, P1 persists throughout the chase in all cells, likely due to inefficient exit from the endoplasmic reticulum (Vogel and Esclamado, 1988; Adema *et al*, 1994; Kobayashi *et al*, 1994; Berson *et al*, 2001, 2003). All bands were precipitable with both HMB50 and αPmel-C, indicating that lumenal and cytoplasmic domains were both present (and linked in the case of Mα/Mβ). Similar results were obtained in a second primary human melanocyte culture and in 1011-mel human melanoma cells. By contrast, only one band

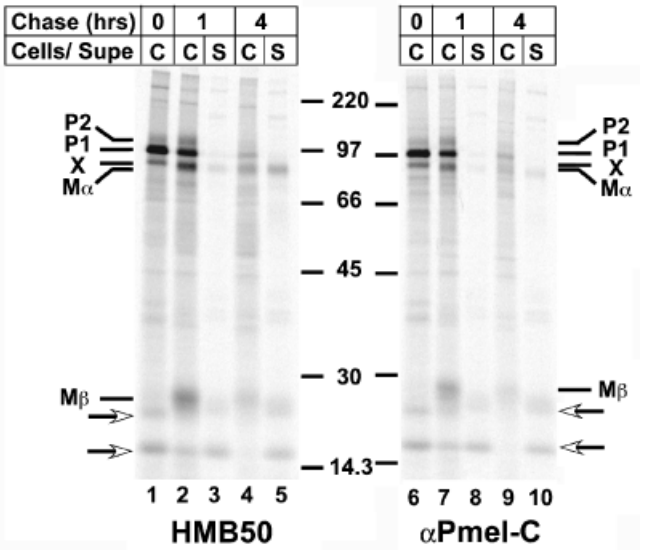


**Figure 1. Processing of Pmel17 and appearance of band X in untransformed human melanocytes.** Primary human foreskin-derived melanocytes were pulse labeled with $^{35}$S-methinonine/cysteine for 30 min and then chased for 0, 1, or 4 h in the presence of excess unlabeled methionine and cysteine. Triton X-100 cell lysates (C) or culture supernatants (S) were immunoprecipitated with antibodies to the lumenal (HMB50, *lanes 1–5*) or cytoplasmic (αPmel-C, *lanes 6–10*) domains of Pmel17, and immunoprecipitates were analyzed by SDS–PAGE and phosphorimaging. The position of molecular weight markers is indicated in the middle. The positions of the core glycosylated precursor (P1), band X (X), the Golgi-processed P2 form, and the proteolytic products Mα and Mβ are indicated. *Arrows*, nonreproducible bands that most likely reflect post-lysis degradation products.

corresponding to the full-length product was observed in immunoprecipitates of pulse-labeled mouse melanocytic cell lines melan-a and melan-a3 (unpublished data). Thus, band X represents a coprecipitating product present in normal and transformed human melanocytes but absent in mouse melanocytes.

**Band X contains the N- and C-termini of Pmel17** To determine whether band X represented a modified form of Pmel17 or a coprecipitating product, we subjected radio-labeled cell lysates to a two-step immunoprecipitation recapture protocol using anti-Pmel17 antibodies. Pmel17 was first immunoprecipitated using αPmel-C or HMB50 under nondenaturing conditions from Triton X-100 lysates of $^{35}$S-methionine pulse-labeled MNT-1 cells (**Fig 2*a***, *lanes 1 and 2*) or cells chased for 2 h in the absence of $^{35}$S-methionine (**Fig 2*b***, *lanes 12 and 13*). Material from these primary immunoprecipitates was then eluted by boiling in the presence of SDS and a reducing agent; this treatment not only dissociates material from the antibodies, but also disrupts any potential complexes containing Pmel17 associated noncovalently or via disulfide bonds to other polypeptides. After removal of the beads, the SDS was diluted and eluates were subjected to a second round of immunoprecipitation using rabbit antibodies directed against peptides corresponding to the N- or C-terminus of Pmel17; because of the SDS treatment, only polypeptides directly recognized by these antibodies should be immunoprecipitated in the second step. As shown in **Fig 2**(***a***, *lanes 3, 4, 6, and 7*), band X is present in the second step immunoprecipitates using both anti-Pmel17 antibodies from lysates of pulse-labeled cells; it was also precipitable using the αPmel-I antibody to an intralumenal peptide (unpublished data). As a control, using lysates from cells chased for 2 h in the absence of radiolabel, Mα or Mβ were precipitable only with the N- or C-terminus-directed antibody,

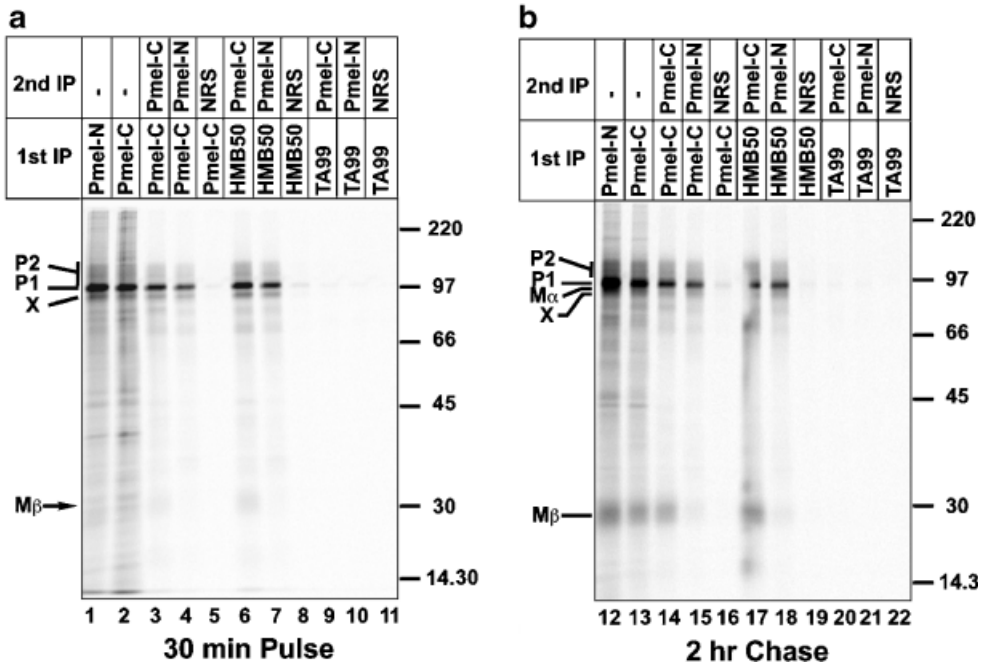


**Figure 2. Immunoprecipitation/recapture of band X using anti-Pmel17 antibodies.** MNT-1 cells were pulse labeled with $^{35}$S-methinonine/cysteine for 30 min and then chased for 0 (*a*) or 2 h (*b*) with excess unlabeled methionine and cysteine. Triton X-100 cell lysates were immunoprecipitated using the anti-Pmel17 antibodies αPmel-N (*lanes 1 and 12*), αPmel-C (*lanes 2–5 and 13–16*), or HMB50 (*lanes 6–8 and 17–19*), or the anti-Tyrp1 antibody TA99 (*lanes 9–11 and 20–22*). Material from the first immunoprecipitate was eluted by boiling with SDS and reducing agent, cooled, diluted with Triton X-100 lysis buffer, and subject to a second round of immunoprecipitation using normal rabbit serum (NRS; *lanes 5, 8, 11, 16, 19, and 22*) or the anti-Pmel17 antibodies αPmel-C (*lanes 3, 6, 9, 14, 17, and 20*) or αPmel-N (*lanes 4, 7, 10, 15, 18, and 21*). Material from the first round (*lanes 1, 2, 12, and 13*) or second round (all other lanes) of immunoprecipitation were fractionated by SDS–PAGE and analyzed by phosphorimaging. The position of molecular weight markers is indicated to the right of each gel, and the migration of the P1, P2, Mα, Mβ, and band X forms of Pmel17 are indicated. Note that (*b*) was exposed for a longer period of time than (*a*) in order to emphasize the Mα and Mβ bands.

respectively (**Fig 2*b***, *lanes 14, 15, 17, and 18*). No bands were recaptured using nonspecific antibodies in the second step (*lanes 5, 8, 16, and 19*) or antibodies to an irrelevant protein in the first step (*lanes 9–11 and 20–22*). From these data, we conclude that band X corresponds to a variant form of Pmel17 and that it contains both the N- and C-termini. We therefore refer to this product herein as Pmel17-s for short form. The data also indicate that Pmel17-s is distinct from the processed Mα cleavage product of Pmel17 (although Mα likely includes a product derived from Pmel17-s; see below).

**Identification of an alternatively spliced, truncated form of Pmel17** The presence of both the N- and C-termini within the single Pmel17-s polypeptide indicates that Pmel17-s cannot be a proteolytic digestion product of full-length Pmel17. Furthermore, the fact that Pmel17-s and full-length Pmel17 continue to migrate differently following digestion with EndoH (Berson *et al*, 2001) excludes the possibility that the two products differ only by the degree of N-linked glycosylation. We therefore hypothesized that Pmel17-s was an alternative Pmel17 translation product derived from a truncated Pmel17 mRNA.

To test for the existence of such an mRNA species, we performed reverse transcriptase–PCR analyses of MNT-1 mRNA using primer sets corresponding to various portions of the Pmel17 coding region (see **Table I**, **Fig 3*a***). As expected, a single band of ≈182 bp was detected using a primer set spanning the coding region for the cytoplasmic domain (**Fig 3*b***, *lanes 13–15*). Using primer sets that spanned only the 3′ third of the Pmel17 coding region, two reverse transcriptase–PCR products could be observed differing by only approximately 20 bp (**Fig 3*b***, *lanes 7–12*). These products were not observed from reverse transcriptase–PCR reactions using mRNA from nonmelanocytic cells (see **Fig 4**) or from preparations of MNT-1 mRNA in which the reverse transcriptase enzyme had not been included (**Fig 3*b***, *lanes 8, 11, and 14*), illustrating the specificity of the signal. These products likely represent the amplified products of the two previously described alternatively spliced mRNA: a less abundant long form, originally cloned and described as Pmel17 (Kwon *et al*, 1991), and a more abundant form, originally cloned and described as gp100 or ME20, lacking 21 bp encoding seven amino acids (Adema *et al*, 1994; Maresh *et al*, 1994) (see **Fig 5*c***). We refer to these two products as Pmel17-l and Pmel17-i for long and intermediate forms, respectively. Even though our reverse transcriptase–PCR reactions were not performed quantitatively, the relative abundance of these forms among our reverse transcriptase–PCR products from MNT-1 mRNA mirrors that previously described in other melanocytic cells in which Pmel17-i is approximately 5-fold more abundant than Pmel17-l (Bailin *et al*, 1996; **Fig 3*b***, *lanes 7–12*). The difference in predicted molecular mass of Pmel17-l and Pmel17-i is less than 700 Da, and is unlikely to be responsible for the ≈5000 Da difference between the unprocessed full-length Pmel17 (P1 form) and Pmel17-s observed in immunoprecipitates. Thus, the difference between Pmel17-s and the longer forms is unlikely due to splice differences in the 3′ half of the Pmel17 coding region.

By contrast, when reverse transcriptase–PCR reactions were performed using primer sets that spanned a longer region encompassing more 5′ coding regions of full-length Pmel17, two bands differing by ≈ 130 bp were observed (**Fig 3*b***, *lanes 1–6*; due to the large size of these products, the 21 bp difference between Pmel17-l and Pmel17-i would not be expected to be resolved). The smaller of these products was less abundant than the larger, representing on average approximately 10% of the total signal. Its size and abundance is consistent with the potential that this band was derived from the mRNA encoding Pmel17-s. Consistently,

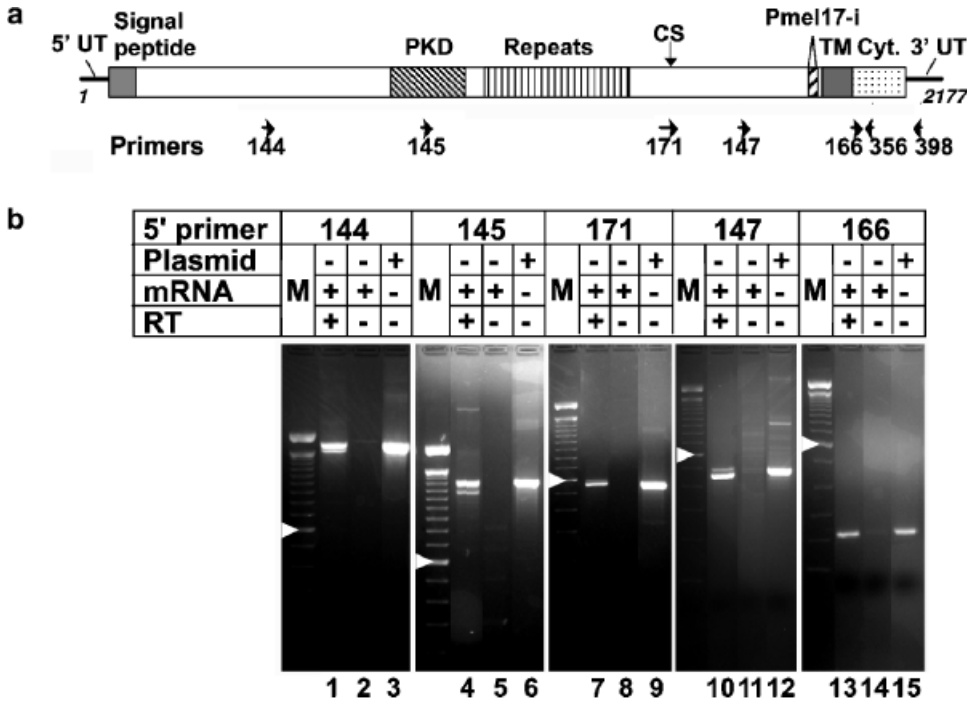


**Figure 3. Detection of Pmel17 mRNA with an internal deletion by reverse transcriptase–PCR of mRNA from MNT-1 cells.** (*a*) Schematic diagram of Pmel17 cDNA and position of primers used in this analysis. The noncoding 5′- and 3′-untranslated regions (5′ UT and 3′ UT) are indicated as solid lines, and the coding region is boxed and divided according to the domain structure of the encoded protein. Indicated are the regions encoding the signal peptide; the lumenal domain PKD homology domain, tandem repeat domain, cleavage site separating the Mα and Mβ fragment (CS), and the peptide excised in Pmel17-i; and the transmembrane (TM) and cytoplasmic (Cyt.) domains. The length of our Pmel17-l cDNA is indicated by nucleotide positions 1 and 2177. *Bottom*, the position of the primers, noted in **Table I**, within the cDNA is indicated, with *arrows* showing the direction of the primer. Not shown is primer 172, which is the reverse complement of 171. (*b*) Reverse transcriptase–PCR reactions from MNT-1 mRNA. MNT-1 mRNA was reverse transcribed using primer no. 398 (*lanes 1, 4, 10, and 13*) or no. 356 (*lane 7*); a parallel sample was incubated under identical conditions in the absence of reverse transcriptase enzyme (*lanes 2, 5, 8, 11, and 14*). Reaction products or 100 ng of purified Pmel17-l plasmid (*lanes 3, 6, 9, 12, and 15*) were subject to 30 cycles of PCR using the indicated forward primers and either no. 356 (*lanes 7–9*) or no. 398 (*all other lanes*) as the reverse primer. M, 100 bp markers; *arrowhead* indicates the 600 bp marker band. Predicted sizes for each of the reaction products for Pmel17-l are 1669, 1265, 524, 444, and 182 bp, respectively.

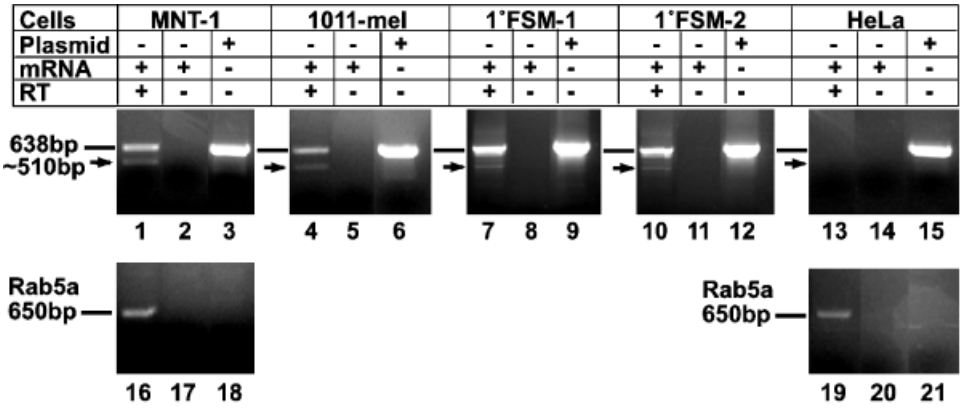


**Figure 4. Broad expression of small Pmel17 mRNA in melanocytic cells by reverse transcriptase–PCR.** mRNA isolated from the melanoma cell lines MNT-1 (*lanes 1–3 and 16–18*) or 1011-mel (*lanes 4–6*), from two different primary foreskin melanocyte cultures (1° FSM-1, *lanes 7–9*; 1° FSM-2, *lanes 10–12*), or from nonmelanocytic HeLa cells (*lanes 13–15 and 19–21*) was reverse transcribed using primer no. 172; parallel reactions were performed identically but with no added reverse transcriptase enzyme (*lanes 2, 5, 8, 11, 14, 17, and 20*). The products of these reactions or 100 ng of plasmid encoding Pmel17-l (*lanes 3, 6, 9, 12, and 15*) were subjected to 30 cycles of PCR using primers no. 172 and no. 145 to amplify a region that would best distinguish the short and long forms of Pmel17 (*lanes 1–15*). The 638 bp reaction product, predicted from the sequence of Pmel17-l, is indicated by a line, and the 510 bp reaction product corresponding to the short form is indicated by an *arrow*. As a positive control, a 650 bp cDNA fragment for Rab5a was amplified using primers no. 319 and no. 321 from RNA isolated from MNT-1 (*lanes 16 and 17*) and HeLa cells (*lanes 19 and 20*); the same reaction was also performed on Pmel17-l plasmid (*lanes 18 and 21*).

the same doublet was observed in reverse transcriptase–PCR products from two primary melanocyte cultures and an additional melanoma cell line, whereas neither product was observed from reverse transcriptase–PCR reactions using RNA from nonmelanocytic HeLa cells or from reactions using melanocytic cell mRNA but lacking reverse transcriptase enzyme (**Fig 4**). Furthermore, reverse transcriptase–PCR reactions using comparable primers for mouse Pmel17 and

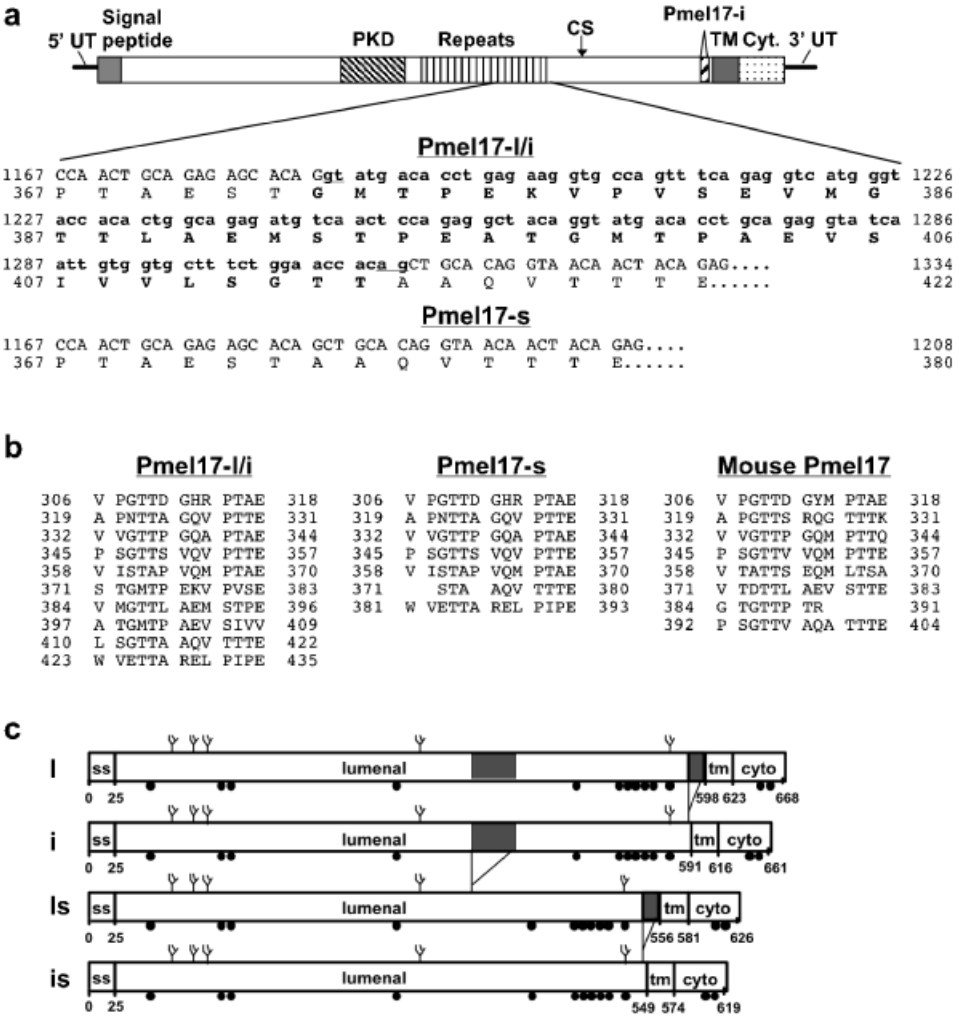


**Figure 5. Sequence analysis of cDNA corresponding to the small Pmel17 mRNA reveals a deletion of part of the internal repeats.** (*a*) Comparison of the sequence of the small and large Pmel17 mRNA. Shown at top is a schematic of the Pmel17-l cDNA (see **Fig 3a** for explanation). The indicated region of Pmel17-l and Pmel17-i is expanded below to show the nucleotide and predicted amino acid sequence. The region in **bold type** is absent from the cDNA isolated for the short form; the nucleotide and predicted amino acid sequence of this region of the short form is indicated at the bottom. Numbers correspond to the nucleotide position of our Pmel17-l cDNA clone and the amino acid positions of the mature protein (with signal peptide removed). (*b*) Comparison of the direct repeat region in Pmel17-l/PMEL17-i with that predicted from the nucleotide sequence of Pmel17-s. The alternative splice removes 3.5 of the 10 imperfect direct repeats. Numbers correspond to the amino acid positions of the mature protein (with signal peptide removed). (*c*) Schematic diagram of four Pmel17 mRNA for which the cDNA were isolated from MNT-1 cells. Lumenal, transmembrane (tm) and cytoplasmic (cyto) domains are indicated. Spliced regions are indicated by gray shading.

mRNA from the mouse melanocytic cell line, melan-a, yielded only a single band corresponding to the expected size of full-length mouse Pmel17 (data not shown), consistent with the failure to detect a Pmel17-s isoform by immunoprecipitation in these cells.

In order to define better the nature of this alternative form of Pmel17, products from reverse transcriptase–PCR reactions from MNT-1 cells were fractionated, excised, subcloned into plasmid vectors, and sequenced. Concordant results from three independent clones derived from two separate reverse transcriptase–PCR reactions using different primer sets revealed that the smaller product lacked 126 bp derived from the middle of the Pmel17 mRNA (nucleotides 1118–1243 of the coding region; **Fig 5*a***). This sequence, encoding more than three of the direct repeats within the Pmel17 lumenal domain (**Fig 5*b***), is encompassed within exon 6 of the Pmel17 gene. The deleted region begins with a "gt" dinucleotide and ends with an "ag" dinucleotide (**Fig 5*a***) but lacks other consensus sites for intron recognition, suggesting that it may represent an infrequently excised, cryptic intron within exon 6. The polypeptide region predicted by the translation of this region (**Fig 5*a***, **bold type**) would have a mass of 4235 Da, consistent with the reduction in relative molecular mass by SDS–PAGE of Pmel17-s compared with full-length Pmel17 (**Figs 1 and 2**). Among the isolated cDNA clones that lacked this 126 bp region, some contained and others lacked the 21 bp region that distinguishes Pmel17-l from Pmel17-i; thus, the two alternative splicing reactions can occur independently and can result in four different Pmel17 mRNA products (**Fig 5*c***).

**Pmel17-s is processed and localized similarly to full-length Pmel17** To confirm that the truncated mRNA encodes Pmel17-s and to compare its biosynthetic characteristics with that of other Pmel17 isoforms, we constructed mammalian expression vectors encoding full-length Pmel17-s, Pmel17-i, and Pmel17-l and expressed the encoded products by transfection in non-melanocytic HeLa cells. As shown in **Fig 6(*a*)**, the product of the putative Pmel17-s cDNA immunoprecipitated from cell lysates of metabolically pulse-labeled, transfected HeLa cells (*lane 5*) comigrated with Pmel17-s from immunoprecipitates of MNT-1

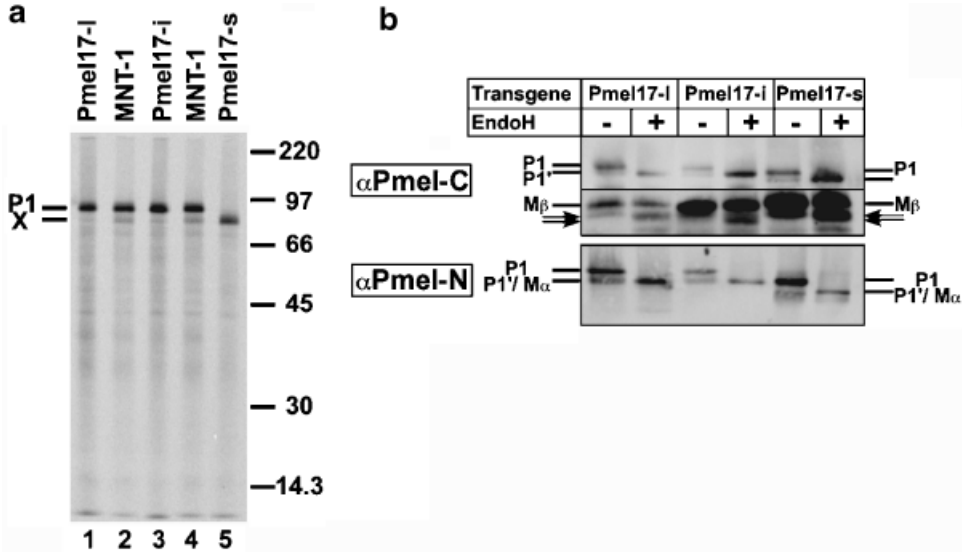


**Figure 6. Expression and processing of Pmel17-s in HeLa cells.** (*a*) Immunoprecipitation of three Pmel17 isoforms from metabolically pulse labeled HeLa cells. MNT-1 cells (*lanes 2 and 4*) or HeLa cells transiently transfected with expression vectors for Pmel17-l (*lane 1*), Pmel17-i (*lane 3*), or Pmel17-s (ls form; see **Fig. 5*c*** *lane 5*) were labeled for 15 min with $^{35}$S-methionine/cysteine. Triton X-100 cell lysates were immunoprecipitated with αPmel-C, fractionated by SDS–PAGE, and analyzed by phosphorimaging. Position of molecular weight markers is indicated to the right, and the positions of the P1 and band X forms of Pmel17 in MNT-1 cells is indicated to the left. (*b*) Western blot analysis of Pmel17 isoforms expressed in HeLa cells. Whole cell lysates of transiently transfected HeLa cells expressing Pmel17-l, Pmel17-i or Pmel17-s were treated or not with EndoH, fractionated by SDS–PAGE on 12% (*upper panel*) or 8% (*lower panel*) polyacrylamide gels, transferred to nitrocellulose using 15% (*upper panel*) or 2% (*lower panel*) methanol, and immunoblotted with antibodies to the cytoplasmic domain (Pmel-C, *upper panel*) or the lumenal domain (Pmel-N, *lower panel*). Relevant portions of the gels encompassing the P1, Mβ, and Mα isoforms, and of EndoH-digested P1 (P1'), as indicated, are shown; no other specific bands were reproducibly observed. Variation in band intensity is due to different transfection efficiencies, which varied from experiment to experiment.

cells (*lane 4*). By contrast, Pmel17-l and Pmel17-i from lysates of pulse-labeled transfected HeLa cells both comigrated with the P1 form observed in MNT-1 cells (compare *lanes 1 and 3* with *lane 2*). Taken together with the data shown in **Fig 2**, this confirms that Pmel17-s is the product of the alternatively spliced, truncated Pmel17 mRNA.

Pmel17 is cleaved in a post-Golgi compartment to Mα and Mβ fragments (Berson *et al*, 2001). To determine whether the internal deletion within Pmel17-s affected the processing of Pmel17 to these fragments, we analyzed transfected HeLa cells by western blotting using antibodies that detect either fragment; whole cell lysates were untreated or treated with EndoH to distinguish core-glycosylated, EndoH-sensitive, pre-Golgi localized isoforms from fully processed, EndoH-resistant, post-Golgi forms. Using αPmel-C to the cytoplasmic domain, a mostly EndoH-resistant Mβ band, in addition to the full-length EndoH-sensitive P1 precursor, was found to accumulate in cells transfected with all three Pmel17 isoforms (**Fig 6*b***, top panel; in data not shown, the migration of EndoH-treated P1 was similar to that of P1 treated with protein N-glycanase F, indicating that all N-linked glycans were susceptible to EndoH digestion). Consistent with previous results (Kobayashi *et al*, 1994; Berson *et al*, 2001), the transient P2 form does not accumulate, whereas the slow exit of Pmel17 from the endoplasmic reticulum is responsible for the accumulation of P1. Whereas the P1 band for Pmel17-s differed in migration from that for Pmel17-l and Pmel17-i, the Mβ band from each migrated identically, consistent with the splice variation occurring within the Mα coding region. Using the αPmel-N antibody to the lumenal domain, a stable EndoH-resistant Mα fragment was found to accumulate in cells expressing each of the three isoforms (note the collapse of EndoH-digested P1 into comigrating EndoH-resistant Mα; **Fig 6*b***, bottom panel). As expected, the Pmel17-s-derived Mα fragment migrated faster than the Pmel17-l- and Pmel17-i-derived Mα fragment. Metabolic pulse/chase and immunoprecipitation analyses also showed similar processing patterns for all three Pmel17 isoforms (data not shown). Thus, alternative splicing does not appear to affect the biosynthetic processing of Pmel17.

When expressed ectopically in nonpigment cells, Pmel17-l localizes to the intralumenal vesicles of late endosomal multivesicular structures and to fibers that form *de novo* upon Pmel17-l expression (Berson *et al*, 2001). To determine whether alternative splicing affects localization, transfected HeLa cells were analyzed by immunofluorescence microscopy for the localization of each of the Pmel17 isoforms. As shown in **Fig 7**, all three isoforms display a similar steady-state localization to vesicular compartments throughout the cytoplasm, as was shown for Pmel17-l. In all three cases, these vesicles overlapped with a subset of Lamp1-containing compartments, and thus correspond to late endosomes or lysosomes; in cells with very high expression, these Lamp1-positive structures were enlarged, and the Lamp1 staining could be observed surrounding the Pmel17 staining (**Fig 7*c,f,i***, insets). Thus, all three isoforms localize similarly in nonpigment cells. We interpret the staining pattern to represent the accumulation of Pmel17 in intralumenal vesicles of late endosomes and/or lysosomes, in which Lamp1 accumulates on the limiting membrane (Berson *et al*, 2001). Given the correspondence of localization to late endosomes in HeLa cells and to melanosome precursors in pigment cells, it is likely that all three isoforms localize similarly in melanocytes as well.

## DISCUSSION

In this study, we have identified a novel splice variant of human Pmel17/gp100, an important constituent of early stage melanosomes and a common target for tumor-directed T lymphocytes in melanoma patients. The expression of this variant by normal and transformed melanocytes has interesting implications for the function of Pmel17 in melanocytes and for the generation of T cell epitopes by melanoma cells.

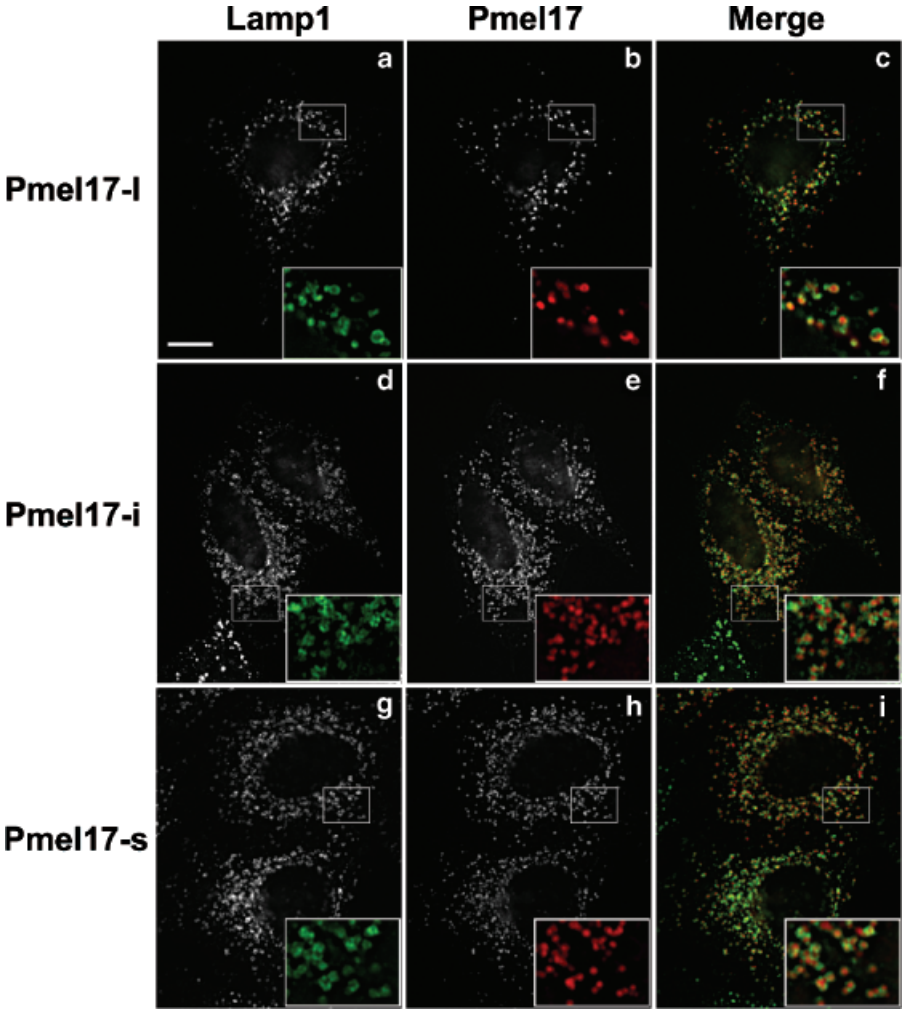


**Figure 7. Immunofluorescence microscopy analyses of the localization of Pmel17-l, Pmel17-i, and Pmel17-s expressed in HeLa cells.** Transiently transfected HeLa cells expressing Pmel17-l (*a–c*), Pmel17-i (*d–f*), or Pmel17-s (*g–i*) were fixed and stained with antibodies HMB50 (IgG2a, to Pmel17) and H4A3 (IgG1, to Lamp1) and isotype-specific, fluorochrome-conjugated secondary antibodies (fluorescein isothiocyanate, anti-γ1; Texas Red, anti-γ2a). Cells were analyzed by immunofluorescence microscopy (IFM), and stacks of images in multiple z focal planes were deconvolved using OpenLab software. Shown are individual fields for Lamp1 (*a,d,g*), Pmel17 (*b,e,h*), and colorized, merged images (*c,f,i*). The boxed region in each panel is magnified × 2.5 at the bottom right of each panel to emphasize the degree of colocalization. *Bar.* (*a*) 10 μm.

**Band X represents the novel Pmel17 splice variant, Pmel17-s** In our previous characterization of Pmel17 in human melanocytic cells (Berson *et al*, 2001), we showed that a large Mα fragment is generated as a result of post-Golgi proteolytic processing of Pmel17. Characterization of Mα, however, was clouded by the presence of a comigrating band, "band X", that was immunoprecipitated from metabolically pulse-labeled cells using anti-Pmel17 antibodies; as Mα and its cognate proteolytic fragment, Mβ, appear only after a chase period, it was difficult to reconcile the appearance of the Mα-like band X prior to Golgi processing. We now clearly identify band X as Pmel17-s, the product of an alternatively spliced Pmel17 mRNA. Because it lacks part of the central region of the lumenal domain of Pmel17, Pmel17-s is directly immunoprecipitable with antibodies to both the N- and C-termini of Pmel17, as well as with additional antibodies to the lumenal domain (**Fig 2**). It is distinguished from Mα based on this immunoreactivity (Mα is not reactive with antibodies to the C-terminus), on its appearance in pulse-labeled cells, and on its sensitivity to EndoH (**Figs 1 and 2**) (Berson *et al*, 2001). A band with similarity to Pmel17-s has been previously observed by other groups (e.g., see Vogel and Esclamado, 1988; Adema *et al*, 1996).

Pmel17-s is generated in melanoma cells and in primary melanocytes (**Figs 1 and 4**), although the relative proportion of Pmel17-s among all Pmel17 products varies from cell to cell and in some cases from experiment to experiment. Although it results from removal of an exonic sequence present in full-length Pmel17 mRNA, Pmel17-s is not consistently generated in HeLa cells transfected with expression vectors for Pmel17-l and Pmel17-i; this was apparent in an earlier study (Berson *et al*, 2001) and allowed us to clearly identify Mα in transfected cells. It does appear to be generated at low levels in Pmel17-l and Pmel17-i transfected HeLa cells in some experiments (e.g., see **Fig 6*a***), suggesting that regulation of the alternative splice may be dependent on cell culture conditions, growth phase of the cells, or expression level. As the Pmel17-s splice was identified both in cDNA that contained and lacked the 21 bp alternative transcript that distinguishes Pmel17-l from Pmel17-i, the two alternative splice reactions must be independently regulated. Thus, human pigment cells can produce at least four distinct Pmel17 gene products by alternative splicing of a single precursor mRNA (**Fig 5*c***).

Although mRNA for Pmel17-s was detected by reverse transcriptase–PCR in all human melanocytic cells tested (**Fig 4**), no comparable product was identified in mouse melanocyte cell lines. This is consistent with the failure to detect a comparable product by immunoprecipitation of metabolically pulse-labeled cells (our unpublished results and Kobayashi *et al*, 1994). Interestingly, although the mouse and human products share considerable homology at both the nucleotide (83%) and amino acid (75%) level, the region surrounding this alternatively spliced region varies significantly in sequence between the two species. This may explain the absence of the Pmel17-s product in the mouse. Given that the mouse Pmel17 gene does not encode the 21 bp insertion found in Pmel17-l (Martínez-Esparza *et al*, 1999), humans have evolved considerable variability in the production of Pmel17 isoforms relative to mice.

**Pmel17-s and the significance of the repeat region** It is intriguing that Pmel17-s lacks several of the characteristic repeats

present within the lumenal domain of Pmel17-l and Pmel17-i (**Fig 5**). This repeat region, first noted by Kwon *et al* (1991), consists of 10 tandem, imperfect repeats of a 13 amino acid sequence rich in glutamine, threonine, glycine, proline, and acidic residues. Our data indicate that the reduction in the number of the repeats has little effect on the post-Golgi processing or ultimate localization of Pmel17 products expressed ectopically in HeLa cells (**Figs 6 and 7**). We speculate that the repeats may have some other function, such as binding to melanin intermediates (Donatien and Orlow, 1995; Chakraborty *et al*, 1996; Lee *et al*, 1996), oligomerization, or fibril formation (Berson *et al*, 2001, 2003). In support of the latter possibility, the amino acid content of the Pmel17 repeats is similar to that of a repeat region within a prion-like protein, Sup35p, in the yeast *Saccharomyces cerevisiae*; these repeats have been shown to be required for the fibril forming activity of Sup35p (Parham *et al*, 2001). We are currently assessing the relative ability of Pmel17 isoforms to induce fibril formation upon expression in nonpigment HeLa cells; preliminary results suggest that the differences are likely to be subtle.

On the other hand, the repeat region of the chicken Pmel17 orthologue, MMP115 (Mochii *et al*, 1991), differs substantially in sequence and general amino acid content from that of mammalian orthologues, perhaps suggesting a role in specific interactions with distinct melanin intermediates. Interestingly, whereas there are 10 direct repeats in full-length human Pmel17 and its bovine orthologue, RPE1 (Kim and Wistow, 1992), the mouse (Kwon *et al*, 1995; Martínez-Esparza *et al*, 1999) and horse (Rieder *et al*, 2000) orthologues, like Pmel17-s, contain only seven. The difference in repeat number between mouse and human correlates with a distinction in the ability to detect post-Golgi processed forms of Pmel17 within melanocytes—these forms are difficult to detect in the mouse (e.g., see Kobayashi *et al*, 1994), yet readily detectable in human melanocytic cells (this study and Berson *et al*, 2001). Perhaps the repeats regulate the kinetics of interactions of Pmel17 with melanins that ultimately mask Pmel17 antibody epitopes (Donatien and Orlow, 1995). Nmb (Weterman *et al*, 1995) and its orthologues QNR71 (Turque *et al*, 1996) and osteoactivin (Safadi *et al*, 2001) are close homologs of Pmel17 but lack any repeat region; comparison of the activities of these proteins with those of the various isoforms of Pmel17 should elucidate a role for the direct repeats.

**Splice variation and anti-melanoma immunity** The existence of a distinct isoform of human Pmel17 has potential relevance for anti-tumor immunity. Pmel17—better known to tumor immunologists as gp100—is a major endogenous and experimental target for melanoma-reactive T lymphocytes; numerous Pmel17-derived epitopes have been found to bind to HLA class I and class II molecules and serve as targets for both $CD8^+$ and $CD4^+$ T cells, respectively (Kawakami *et al*, 2000; Overwijk and Restifo, 2000; Slingluff *et al*, 2000). The most broadly recognized T cell epitopes cluster either in the amino-terminal region of the lumenal domain or downstream of the repeat region (Kawakami *et al*, 1998b; Castelli *et al*, 1999; Bullock *et al*, 2000; Cochlovius *et al*, 2000; Touloukian *et al*, 2000; Kierstead *et al*, 2001; Kobayashi *et al*, 2001). Nevertheless, the region that is deleted from Pmel17-s spans two consensus binding epitopes for HLA-DR molecules, including one with very high affinity for both HLA-DRB1*0404 and DRB1*0401 (Kierstead *et al*, 2001), and another with broad HLA-DR binding activity (Cochlovius *et al*, 2000). The latter epitope was capable of eliciting a T cell proliferative response from several healthy donors (Cochlovius *et al*, 2000). Thus, altered expression of Pmel17 isoforms may affect T cell immune responses. Furthermore, the novel peptide sequence generated by Pmel17-s could potentially encompass a neorecognition element for certain HLA-DR molecules. That such a neoepitope could exist is underscored by the finding that tumor-infiltrating $CD8^+$ T cells derived from a melanoma patient recognized an HLA-A24-binding peptide derived from an intronic sequence (Robbins *et al*, 1997). The existence of such an epitope from Pmel17-s could potentially explain the reactivity of Pmel17-specific tumor-infiltrating T cells from a melanoma patient that were unable to recognize autologous antigen-presenting cells pulsed with peptides derived from the entire sequence of Pmel17-l and Pmel17-i (Robbins *et al*, 2002).

*We thank G. Raposo and A. Theos for helpful discussions and critical reading of the manuscript, A. Yoshino for assistance with the reverse transcriptase–PCR reactions, and M. Herlyn and R. Finko for gifts of the primary melanocyte cultures. This work was supported by National Institutes of Health grants no. R01 EY012207 from the National Eye Institute and R01 AR048155 from the National Institute for Arthritis and Musculoskeletal and Skin Diseases.*

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