INDUCTION OF PIGMENTATION IN MOUSE FIBROBLASTS BY EXPRESSION OF HUMAN TYROSINASE cDNA

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Cells of the melanocyte lineage are distinguished by their capacity to synthesize the pigment melanin. Production of melanin is primarily regulated by the enzyme tyrosinase (monophenol, 3,4-dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1), and it is presumed that melanin synthesis can be regulated at a number of levels that control both the amount of melanin synthesized and the type of melanin produced. Melanin synthesis occurs principally in specialized organelles, the melanosomes. Thus, the synthesis of melanin is usually restricted to melanocytic cells that contain melanosomes.

In this report, we describe isolation of a full-length cDNA clone encoding human tyrosinase by using a probe homologous to the Pmel 34 cDNA sequence described by Kwon et al. (1). We have transfected and expressed this new human tyrosinase cDNA clone in mouse fibroblasts, and have induced pigmentation in a cell type that does not normally synthesize melanin. Levels of tyrosinase activity in transfected fibroblasts were equivalent to tyrosinase levels in highly pigmented human melanoma cell lines. These tyrosinase-positive fibroblast cell lines demonstrate that melanin synthesis can take place in cells that do not have melanosomes and, therefore, provide a tool for studying the regulation, transport, and processing of tyrosinase.

Materials and Methods

Cell Culture and Cell Lines. Melanoma cell lines were established as previously described (2). TK^- L929 cells (mouse fibroblasts) (3) were used for transfection experiments. Cell lines were maintained in Eagle's MEM supplemented with 2 mM glutamine, 0.1 mM nonessential amino acids (aa)[1], 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10% FCS (complete medium). Cells were passaged with trypsin (1 mg/ml) and EDTA (0.2 mg/ml). All cultures were checked regularly for the presence of mycoplasma and contaminated cultures were discarded.

EM. Cell pellets were fixed in Karnofsky's fixative overnight, rinsed in PBS for 1 h, and then post-fixed for 1 h in 1% osmium tetroxide-PBS solution. Cell pellets were dehydrated in graded ethyl alcohol followed by propylene oxide, and embedded in Maraglas-D.E.R. 732 epoxy resin (Dow Corning Corp., Midland, MI). For orientation, 1-μm thick sections were

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[1] *Abbreviations used in this paper:* aa amino acid; DOPA, dihydroxyphenylalanine; NET, NaCl/EDTA/Tris.

stained with borate-buffered 1% toluidine blue. Thin sections were stained with uranyl acetate followed by lead citrate and were examined with an electron microscope (410 LS; Phillips Electronic Instruments, Inc., Mahwah, NJ).

cDNA Library and Screening. A cDNA library was constructed from 3 μg of poly(A)$^+$ selected mRNA (4) prepared from the human melanotic melanoma cell line SK-MEL-19 (2). Full-length cDNA was synthesized, rendered blunt ended using Klenow enyzme, and tailed with Eco RI linkers (New England Biolabs, Inc., Beverly, MA) (5). The cDNA was then size fractionated on Ultrogel Aca 34 (Pharmacia Fine Chemicals, Piscataway, NJ) (6). cDNA molecules >800 bp were used to construct a library of 3×10^5 recombinants in the λ phage vector gt10 (7). For screening, a 50-base oligonucleotide probe (50-mer, shown below) based on the 5′ terminal coding region of the human tyrosinase Pmel 34 cDNA clone (1) was used: 5′ GTTCTTAGAGGAGACACAGGCTCTAGGGAAAATGGCCAGCGGAGGTCTGGA 3′.

The oligonucleotide was synthesized on a DNA synthesizer (310 A; Applied Biosystems, Inc., Foster City, CA). The probe was end labeled with γ-[^{32}P]ATP and T4 polynucleotide kinase (4). Prehybridization and hybridization were carried out at 48°C for 4 and 18 h, respectively, in 6× NaCl/EDTA/Tris (NET) (1× NET is 0.15 M NaCl, 1 mM EDTA, and 15 mM Tris-HCl, pH 8), 0.1% SDS, and 5× Denhardt's solution (0.1% BSA, 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone), and 100 μg/ml of denatured salmon sperm DNA. Duplicate filters were washed in 6× NET, 0.1% SDS at room temperature, followed by stringent washes at 55°C and 60°C. The filters were then autoradiographed for 4 h at −70°C.

DNA Sequencing. Plaque-purified phage DNA was restricted with Eco RI, and cDNA inserts were subcloned into the plasmid vector pUC 18 (8). Recombinant plasmids and deletion subclones subsequently obtained by digestion with exonuclease III/Mung Bean nuclease (9) were sequenced by the dideoxynucleotide chain termination method (10).

Northern Blot Analysis. Poly (A)$^+$-mRNA (4 μg) was fractionated on 1% formaldehyde denaturing agarose gels (4), transferred to Gene Screen Plus membranes (New England Nuclear, Boston, MA), and hybridized to a ^{32}P-labeled cDNA probe. The filters were washed twice at room temperature in 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7) and 1% SDS, then stringent washes were carried out at 55°C in 1× SSC, 1% SDS, and at 65°C in 0.1× SSC, 1% SDS, for 15 min each.

Transfection Experiments. The cDNA inserts were subcloned into the Eco RI site of the expression vector pcEXV-3, which allows expression of cDNA under the control of SV40 early region promoter and enhancer sequences (11). Expression plasmids containing cDNA inserts in opposite orientations (5′→3′ or 3′→5′) were constructed. Sense and antisense oriented plasmids were designated pcTYR and pcTYW, respectively. L929 cells were cotransfected by the calcium phosphate precipitation technique (12) with pUC 18, pcTYW or pcTYR, the pSV2 *neo* plasmid, and high molecular weight carrier DNA from L929 cells. Selection of transfectants was started on day 3 after transfection with 1 mg/ml of the antibiotic G418 (Sigma Chemical Co., St. Louis, MO). Complete medium with G418 was replaced every 3 d, and colonies appearing on days 10–14 were isolated using cloning rings and were then expanded. The mouse origin of transfected cell lines was confirmed by positive anti-mouse Ig mixed hemadsorption assays using H100-5R28, an mAb directed against H-2K^k (mouse MHC class I antigens) (13), and lack of reactivity with mAb M3-68 (14) or AJ2 (15), which recognize virtually all human melanoma cells but not L929 cells (data not shown).

Serological Reagents and Assays. CF21 (IgG1) and TA99 (IgG2a) are mAbs, which have been previously described (16), that recognize distinct antigens in human melanosomes. The mAb 2G10 (IgG2a) (17) was a generous gift from Dr. P. G. Natali, (Regina Elena Cancer Instituto, Rome, Italy). This antibody recognizes a 75-kD intracellular glycoprotein of pigmented melanotic cells (17). mAb AJ2 (IgG1) recognizes the β subunit of human integrin molecules (15, 18). Rabbit antityrosinase antiserum was raised by immunization with purified mouse tyrosinase (19). Briefly, tyrosinase was purified by DEAE ion exchange chromatography followed by sequential discontinuous PAGE. The anti-mouse Ig hemadsorption assays and the indirect immunofluorescence studies were performed as described (2, 20).

Immunoprecipitations. Cells were labeled with ^{35}S-methionine (ICN Radiochemicals, Irvine, CA) for 16 h in methionine-free complete medium containing 2% dialyzed FCS, and lysed in 50 mM Tris, 5 mM EDTA, 0.5% NP-40, 1 mM PMSF. The lysates were precleared

twice by incubation with 5 μg/ml of protein A-Sepharose (Pharmacia Fine Chemicals) for 30 min at 4°C. Immunoprecipitations were performed by incubating the lysates with antibodies, followed by addition of protein A-Sepharose. The immunoprecipitates were extensively washed and analyzed for molecular size by SDS/PAGE (21) under reducing conditions.

Tyrosinase Activity and Melanin Assays. Cells were solubilized in PBS, 1% NP-40, pH 6.8, and centrifuged to obtain clear supernatants. Tyrosine hydroxylase activity was assayed using a modification of the method described by Pomerantz (22). Briefly, the reaction mixture contained 1 μCi/ml [^{3}H]tyrosine (54.2 Ci/mMol) in PBS, 1% NP-40, 0.1 mM L-tyrosine, and 0.1 mM L-dihydroxyphenylalanine (L-DOPA). The reaction was carried out at 37°C for 1 h, and terminated by addition of 0.2 ml of a charcoal suspension (100 mg/ml in 0.1 M citric acid). After 30 min on ice, the samples were centrifuged and an aliquot was counted in a scintillation counter (LS 9000; Beckman Instruments, Inc., Fullerton, CA). All assays were performed in duplicate. Controls included ^3H_2O release measured in lysates from the human renal carcinoma cell line SK-RC-7 and reaction mixture in PBS, 1% NP-40 alone. Specific tyrosinase activity was calculated as follows: (^3H_2O release by test cell lysate) − (^3H_2O release by control reaction mixture in PBS). Protein concentrations were determined by the Bradford's dye binding method using the Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA). For melanin assays, 3×10^6 cells were solubilized in 0.5 ml Protosol (New England Nuclear) and kept on ice for 2 h. An absorption baseline was established using Protosol, and absorption spectra for cell extracts were determined between 320 and 450 nm and compared against a melanin control, 100 μg/ml, in Protosol.

Results

Isolation and Sequencing of the cDNA Clone BBTY-1. cDNA clones were isolated from a λ gt10 library derived from the pigmented human melanoma cell line SK-MEL-19 (see Materials and Methods). 10^5 recombinant cDNA clones were screened and four reactive clones were plaque purified. The four cDNA inserts were subcloned into the plasmid vector pUC 18 and clones were designated pBBTY-1, -2, -3, and -4. Two clones, pBBTY-1 and pBBTY-2, each containing cDNA inserts of 2 kb, had restriction maps identical to each other and to that of Pmel 34, reported by Kwon et al. (1) (digested with Bgl II, Hpa II, Msp I, Nco I, Pvu II, and Taq I). The cDNA inserts in clones pBBTY-3 and pBBTY-4 were 1.7 and 1.8 kb, respectively. The restriction map of pBBTY-3 was different from those of pBBTY-1 and pBBTY-2 downstream of position 960 (a Pvu II restriction site). pBBTY-1 was subsequently sequenced and used for further experiments.

The nucleotide sequence of BBTY-1 (Fig. 1) contained a single open reading frame of 1,593 residues capable of encoding a 531-aa polypeptide with a derived molecular mass of 60.37 kD. A leader peptide of 19 aa was assigned to positions -19 through -1 (23). The processed core protein was predicted to have a molecular mass of 58.11 kD. Seven potential *N*-glycosylation signals (Asn-X-Ser/Thr) were predicted at positions 69, 94, 144, 213, 273, 320, and 354. Based on a hydrophobicity plot, according to the method of Kyte and Doolittle (24), a transmembrane region was predicted within a highly hydrophobic domain between aa positions 470 and 490. There was a 318 base 3′ noncoding region that contained an atypical polyadenylation signal, AATTAAA (25). The nucleotide and aa sequences of BBTY-1 were nearly identical to the sequence of the Pmel 34 cDNA. (1). However, BBTY-1 contained an additional upstream 5′ sequence, including a potential initiation codon not present in Pmel 34 (bases 1–7). There were also differences in the predicted aa sequence of BBTY-1 at positions 25–28, 162, 291, 356–361, 385, 478, and 503–512. The predicted molecular size of the processed protein coded by BBTY-1 was smaller than the processed

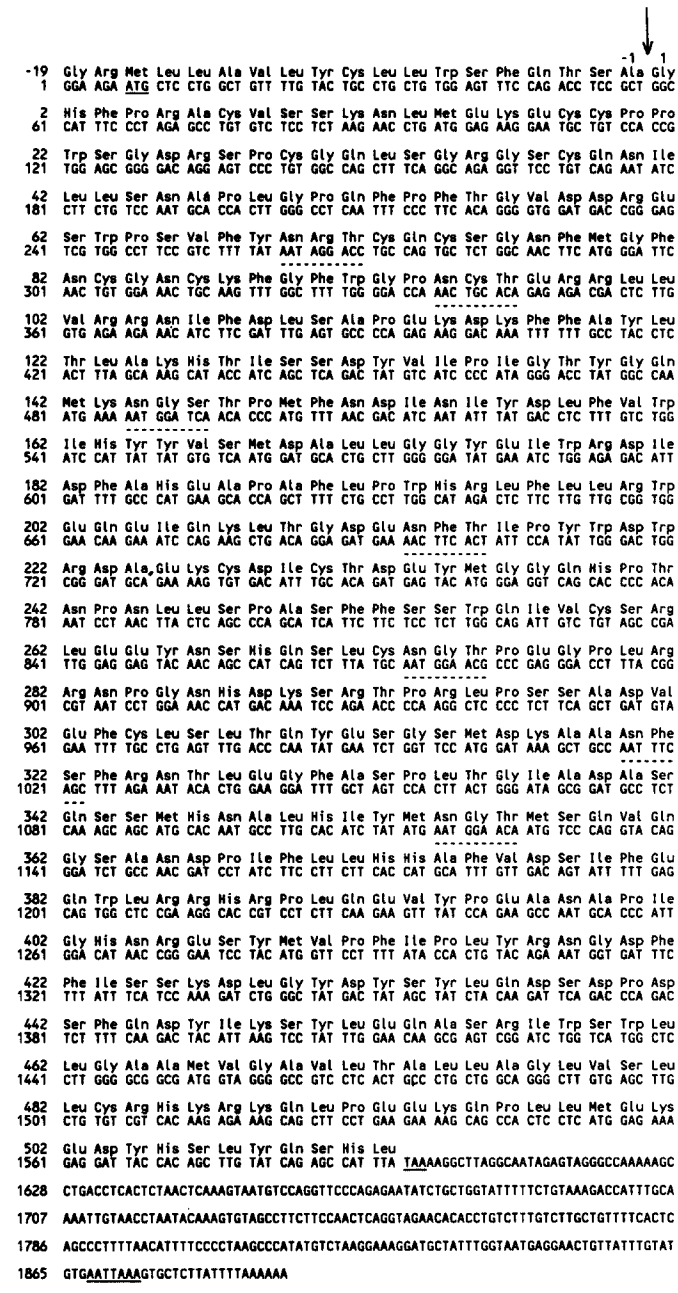

FIGURE 1. Nucleotide and predicted amino acid sequence of BBTY-1 cDNA. The nucleotide sequence is numbered in the 5′ to 3′ direction. Residues of a predicted signal peptide are indicated by negative numbers, and a cleavage site by a vertical arrow. Termination site (TAA) and polyadenylation signal (1869–1875) are underlined. Potential glycosylation sites are designated by dashed lines. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00819.

protein predicted from Pmel 34 (62.16 kD). Based on this sequence analysis, BBTY-1 was a candidate for a full-length cDNA clone encompassing a complete coding region.

Transcription of BBTY-1 in Human Melanoma Cells. BBTY-1 cDNA was used to detect mRNA transcripts in Northern blot analysis of a panel of melanoma cell lines, including those known to express tyrosinase activity as well as tyrosinase-negative melanomas. The major transcript detected was 2.4 kb, but a weaker signal was seen at 4.7 kb (Fig. 2). Three groups of melanomas were observed based on Northern blot analysis using poly $(A)^+$-selected RNA (data not shown). (*a*) mRNA was detected in nine pigmented melanomas that express tyrosinase activity; (*b*) no mRNA was detected in five nonpigmented, tyrosinase-negative melanomas; and (*c*) mRNA was detected in three nonpigmented, tyrosinase-negative melanomas. There was little or no difference in the intensity of mRNA signal detected in group *c* vs. *a*. No transcript was detected in mRNA from the B cell lymphoma cell line Daudi or from the T cell leukemia cell line HUT-78.

Melanin Synthesis in L929 Cells Transfected with BBTY-1. BBTY-1 was transfected into L929 mouse fibroblasts using the expression vector pcEXV-3 (11). L929 cells transfected with pcTYR (sense orientation) were designated LpcTYR. Control cells transfected with pcTYW (antisense orientation) were designated LpcTYW, and, with the plasmid pUC 18, were designated LpC. LpcTYR cells contained pigment, while no pigmentation was detected in LpcTYW, LpC, or untransfected L929 cells. As shown in Fig. 3, the cell pellets of LpcTYR clones were dark brown in contrast to the nonpigmented pellets of LpcTYW and LpC cultures.

Cell pellets of LpcTYR were more deeply pigmented when cultures were harvested at confluency. LpcTYR clones have continued to produce pigment for >5 mo in continuous culture. To confirm that the pigment in LpcTYR has the characteristics of melanin, absorption spectra of cell extracts from LpcTYR and control L929 cells were compared with those of extracts of the pigmented melanoma cell line SK-MEL-19 and purified melanin. LpcTYR and SK-MEL-19 extracts and melanin had identical patterns of absorption, with broad absorption from 360 to >450 nm; this absorption pattern was not observed with L929 cell extracts (data not shown). The absorption patterns by extracts of LpcTYR and SK-MEL-19 and melanin standard were identical to the previously described absorption spectra for melanin (26).

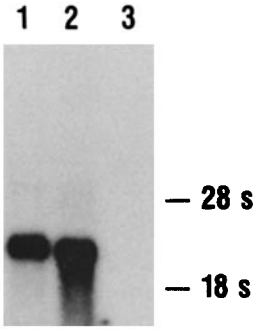

FIGURE 2. Northern blot analysis of poly $(A)^+$-selected RNA (4 μg/lane) from two pigmented melanoma cell lines that express tyrosinase and the B cell lymphoma cell line Daudi. The blot was hybridized with the ^{32}P-labeled insert of pBBTY-1. Lanes *1*, SK-MEL-23 melanoma; lane *2*, SK-MEL-19 melanoma; lane *3*, Daudi.

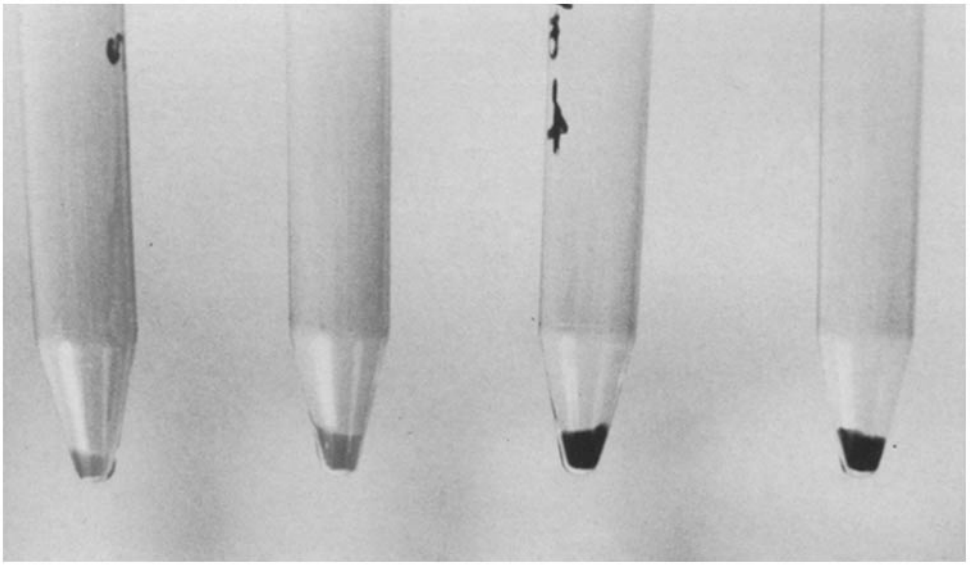

FIGURE 3. Cell pellets of L929 cells transfected with sense BBTY-1 (LpcTYR cell line), antisense BBTY-1 (LpcTYW cell line), or pUC 18 plasmid (LpC cell line). LpcTYR and LpcTYW cell lines were transfected with BBTY-1-inserted sense or antisense into the expression vector pcEXV3. LpcTYR-1 and LpcTYR-2 are subclones of LpcTYR. (*1*) LpC cell pellet (nonpigmented); (*2*) LpcTYW (nonpigmented); (*3*) LpcTYR-1 (pigmented); and (*4*) LpcTYR-2 (pigmented).

Small clusters of cell containing dark cytoplasmic inclusions were observed throughout the LpcTYR culture by light microscopy (Fig. 4). These clusters of cells always comprised a minority of the culture population. Occasional black round cells were detected floating in the tissue culture medium, perhaps related to cytostatic or cytotoxic effects of melanin by-products, and the prevalence of these cells increased as the culture reached confluency. Transmission EM revealed that LpcTYR cells, but not control LpC cells, had cytoplasmic membrane-bound vesicles (Fig. 5) containing electron-dense material consistent with melanin. There was no evidence of melanosomal structural elements within LpcTYR cells or LpC cells.

Tyrosinase Activity in L929 Cells Expressing BBTY-1. To confirm that the BBTY-1 product was human tyrosinase, tyrosine hydroxylase activity was measured in protein extracts of subclones of LpcTYR, LpcTYW, and LpC. Cell extracts from two subclones of LpcTYR, designated LpcTYR-1 and LpcTYR-2, expressed levels of tyrosinase activity that were comparable with levels in the pigmented human melanoma cell line SK-MEL-19 (Fig. 6). In contrast, extracts of LpcTYW and LpC contained no detectable tyrosinase activity.

Analysis of Expression of Melanosomal Antigens in LpcTYR Cells. LpcTYR-2, SK-MEL-19 melanoma cells, and control L929 cells were metabolically labeled with ^{35}S-methionine and cell extracts were immunoprecipitated with rabbit antityrosinase antiserum or mAb TA99 (which detects the melanosomal antigen gp75). In addition,

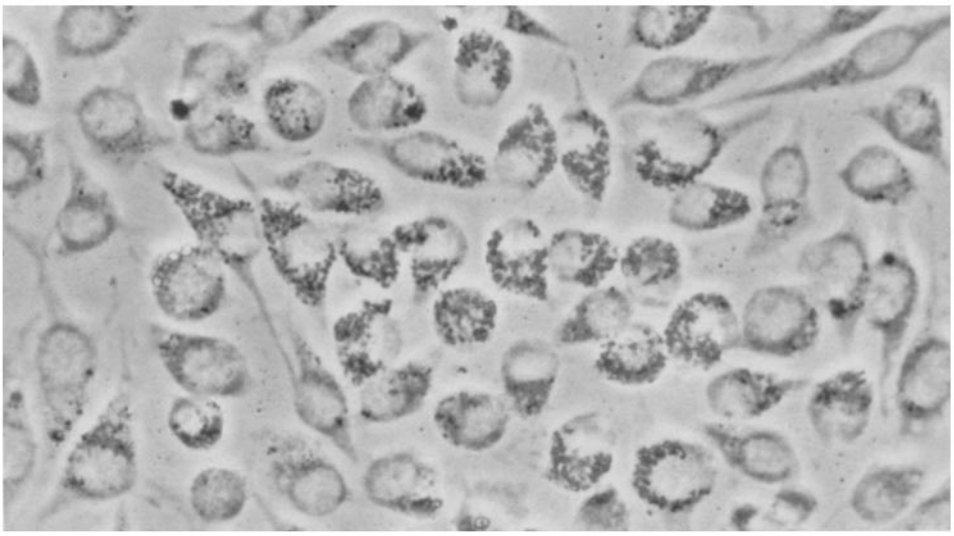

FIGURE 4. LpcTYR cells in culture. A nest of cells in the middle of the field contains large, pigmented cytoplasmic granules. (×320).

mAb 2G10 (from Dr. Pier Natali, Regina Elena Instituto, Rome), which is also directed against an intracellular 75-kD antigen expressed by pigmented melanoma cells (17), was tested. Antityrosinase antiserum detected a 75-kD protein in LpcTYR-2 cells and a protein of the same size in SK-MEL-19 melanoma cells (Fig. 7). The molecular size

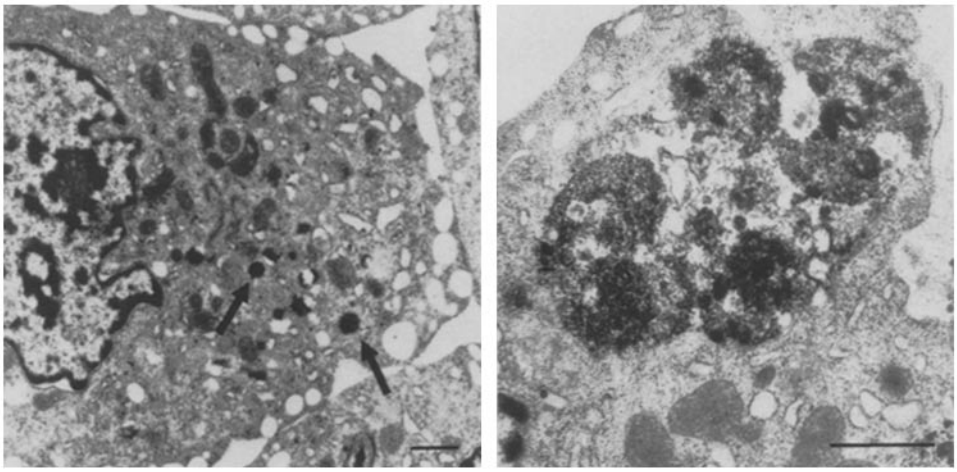

FIGURE 5. Transmission electron micrographs of segments of LpcTYR cells. (*A*) Cytoplasmic membrane-bound vesicles containing electron-dense material are indicated by arrows. One scale bar represents 1 μm. (×8,400). (*B*) Higher magnification field of a cytoplasmic membrane-bound vesicle containing pigment. One scale bar represents 1 μm. (×16,800).

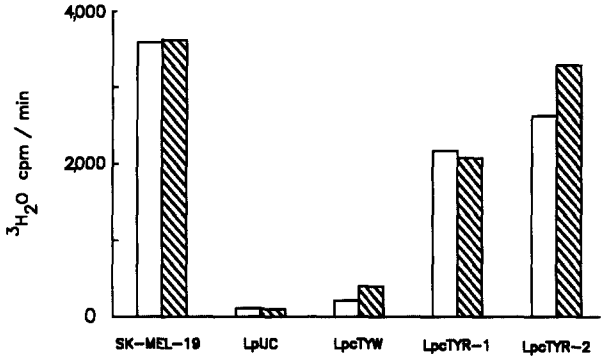

FIGURE 6. Expression of tyrosinase activity in cell extracts from: SK-MEL 19 melanoma; Lpc cells (transfected with pUC 18 plasmid); LpcTYR-1 cells (transfected with a BBTY-1 sense construct); LpcTYR-2 cells (transfected with a BBTY-1 sense construct); and LpcTYW cells (transfected with a BBTY-1 antisense construct). Tyrosine hydroxylase activity is expressed as cpm ^3H_2O/min/mg protein (□) or cpm ^3H_2O/min/5 × 10^6 cells (▨).

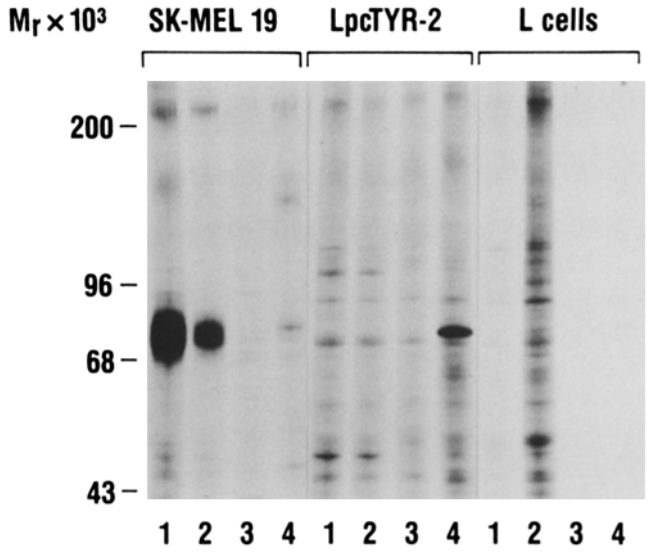

FIGURE 7. Immunoprecipitation of lysates from ^{35}S-methionine metabolically labeled SK-MEL-19 melanoma cells, LpcTYR-2 cells expressing BBTY-1, and L929 cells. Lane *1*, mAb TA99; lane *2*, mAb 2G10; lane *3*, control rabbit sera; and lane *4*, rabbit antityrosinase antisera. A 75-kD band is detected in SK-MEL-19 (with TA99, 2G10, and antityrosinase) and LpcTYR-2 cells (with antityrosinase). Molecular weight standards: Myosin M chain (200 kD); phosphorylase (96 kD); BSA (68 kD); and OVA (43 kD).

of tyrosinase in LpcTYR-2 and SK-MEL-19 cells corresponded to the size of glycosylated tyrosinase. A very faint band at ~75 kD was inconsistently detected in L929 cells with antityrosinase antiserum; this likely represents a crossreaction of polyclonal sera to a nontyrosinase molecule in L929 cells, since no tyrosinase activity or tyrosinase transcript was detected in these cells and cold lysates from L929 cells did not block immunoprecipitation of tyrosinase from LpcTYR-2 (data not shown).

No specific bands were detected by either mAb TA99 or mAb 2G10 in LpcTYR-2 extracts, although both antibodies precipitated a broad 75-kD band from melanoma SK-MEL-19 lysates. These results were confirmed using immunofluorescence assays. Neither mAb TA99 nor 2G10 stained LpcTYR cells but both reacted with SK-MEL-19 cells (Fig. 8). In addition, mAb CF21, directed against a melanosomal antigen of unknown molecular size, did not react with LpcTYR but stained SK-MEL-19 (Fig. 8). We conclude that mAbs TA99, CF21, and 2G10 identify antigens distinct from tyrosinase encoded by the BBTY-1 cDNA clone.

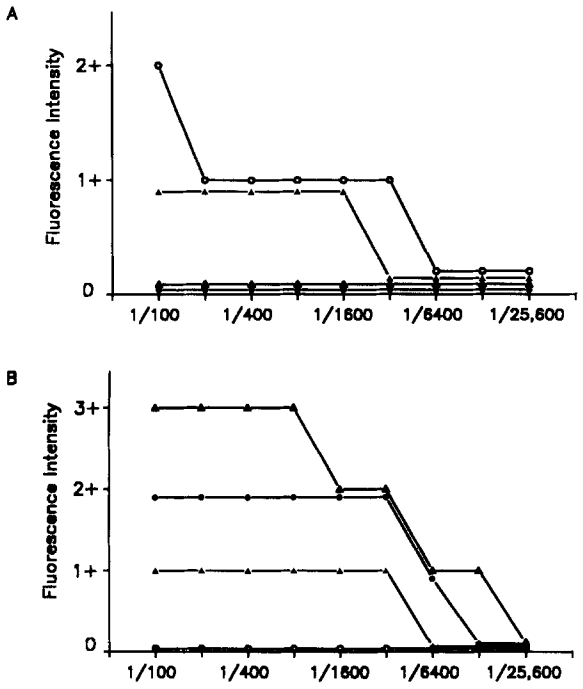

FIGURE 8. Indirect immunofluorescence assays for antigen expression by: (*A*) LpcTYR-2 cells expressing BBTY-1; and (*B*) SK-MEL-19 melanoma cells. mAb TA99 (anti-gp75) (Δ); mAb CF21 (antimelanosomal antigen) (●); mAb H100-5R28 (anti-H-2^k) (O); and mAb AJ2 (antiintegrin; positive control) (▲).

Discussion

Tyrosinase catalyzes the *o* hydroxylation of monophenols and oxidation of *o*-diphenols to *o*-quinones. In melanocytic cells, tyrosinase enzymatically converts tyrosine to DOPA, and DOPA to dopaquinone, leading to the spontaneous formation of the complex mixture of pigments known as melanin (27). The later steps in this pathway are not well characterized, and it has been suggested that a number of other factors, both catalytic and inhibitory, may regulate melanin synthesis and influence the species of melanin formed (28, 29). The complexity of pigment expression has been further highlighted by genetic studies in the mouse where >50 loci have been found to influence coat color (30). Thus, it is possible that a number of gene products, most not yet identified, can play a role in melanogenesis.

It is remarkable that transfected L929 fibroblasts not only stably expressed tyrosinase activity but were able to produce and package melanin. Melanin precursors are cytotoxic, and it has been presumed that melanocytic cells contain mechanisms, perhaps located within melanosomes, that protect from the effects of toxic intermediates. We suspect that melanin precursors were in fact cytotoxic in transfected L929 cells, and that cells producing substantial amounts of pigment were destined to die, based on the following observations: (*a*) only a subpopulation of transfected cells contained pigmented vesicles; (*b*) deeply pigmented, nonviable cells were observed floating in the supernatant of transfectant cultures; and (*c*) when transfected cells were cryopreserved and then thawed, pigmented cells were not initially detected but eventually repopulated the culture.

We have not yet analyzed in detail the synthesis or processing of human tyrosinase in transfected L929 cells. From our preliminary studies, it appears that human tyrosinase is glycosylated to a form that is identical in size to fully processed tyrosinase expressed in human melanocytic cells. It is likely that human tyrosinase was processed through the Golgi apparatus in L929 cells and transported to or remained in vesicles arising from the *trans*-Golgi. The nature and destination of these vesicles is not known. It is interesting to speculate that these vesicles might be precursors of melanosomes but that formation of melanosomes would depend on the products of other specialized genes.

The expression and regulation of tyrosinase has been the subject of extensive studies, but the formal identification of the gene that codes for tyrosinase has not been straightforward (reviewed in reference 31). Two distinct, and only distantly related, genes have been proposed as candidates for mouse tyrosinase, based on detection of mRNA of these genes in melanocytic cells and reactivity of the protein product with antibodies against tyrosinase (32, 33). Neither gene, however, was demonstrated directly to code for a product with tyrosinase activity. It is likely that antibodies used to detect the products of putative tyrosinase cDNA clones reacted with other molecules that copurified with tyrosinase. This situation was recently clarified by the identification of the mouse tyrosinase gene by Müller et al. (34) who isolated a cDNA clone, pmctyrl, that coded for transient expression of tyrosinase activity in transfection assays. No pigment synthesis was reported in transfected cells, possibly because assays were performed only shortly after transfection, because the recipient cells were different (an amelanotic melanoma and a breast carcinoma cell line), or because levels of tyrosinase activity appeared to be much lower than in mouse fibroblasts transfected with BBTY-1.

The candidate for the human tyrosinase gene, designated Pmel 34, has been reported by Kwon et al. (1). Kwon et al. (35) also recently described a mouse cDNA, MTY811C, isolated using Pmel 34. The gene product encoded by MTY811C was predicted to be 81% homologous to the protein encoded by Pmel 34. Both the human Pmel 34 and the mouse MTY811C correspond to the human counterpart of the mouse pmctyrl gene, and in fact, the pmctyrl clone was also isolated by screening a cDNA library from mouse melanoma cells with the Pmel 34 cDNA. The Pmel 34 cDNA clone was detected by screening a cDNA library with polyclonal antisera raised against hamster tyrosinase. Pmel 34 has been mapped to the c (albino) locus in the mouse, the presumed site of the tyrosinase structural gene or a gene that regulates tyrosinase expression. The nucleotide and predicted aa sequences of BBTY-1 and Pmel 34 are nearly identical. BBTY-1 contains an initiation codon that is not present in Pmel 34, and there are minor differences in nucleotide and predicted aa sequences. It is possible that some of these differences represent genetic polymorphism or somatic mutations (related to the source of cell types used to isolate cDNA, i.e., melanoma cells for BBTY-1 vs. melanocytes for Pmel 34). It is interesting to note that where there are distinct differences in sequences between BBTY-1 and Pmel 34, the sequence of BBTY-1 is very close or identical to the mouse pmctyrl tyrosinase sequence (e.g., aa 356–361 and 385).

Multiple transcripts of the tyrosinase gene have been found in mouse melanoma cells (36). The remaining transcripts are generated by alternative splicing leading to deletion of internal sequences, presumably by exon skipping or by selection of

internal splice sites. When these alternative transcripts have been expressed, they have not been found to encode active tyrosinase (34, 36). The BBTY-1 cDNA represents the human counterpart of the mouse pmctyr1 transcript. Another cDNA clone that we isolated, BBTY-3, differs from BBTY-1 in its 3′ restriction map, possibly corresponding to an alternative transcript of the human tyrosinase gene.

We have asked what is the relationship of tyrosinase to the melanosomal/cytoplasmic antigens recognized by mAbs 2G10, TA99, and CF21. It has been shown that mAb 2G10 immunodepletes tyrosinase activity (37) and, therefore, possibly recognizes a molecule with tyrosinase activity. However, mAb 2G10 did not react with human tyrosinase encoded by BBTY-1, suggesting that mAb 2G10 recognizes a distinct molecule from the gene product of BBTY-1. TA99 mAb recognizes an acidic 75-kD glycoprotein (38), and the antigen recognized by TA99 is a candidate for tyrosinase, based on its expression in melanosomes, its molecular size, and charge. The finding that mAbs TA99 and CF21 did not react with L929 transfectants provides evidence that they do not recognize determinants coded for by the BBTY-1 human tyrosinase molecule. Further data suggest that mAb TA99 does not recognize tyrosinase: (*a*) mAb TA99 does not precipitate tyrosinase activity from melanoma cell extracts (39, 40); (*b*) the TA99 antigen, gp75, is generally coexpressed with tyrosinase activity, but there are examples of gp75$^+$ melanoma cell lines that do not express tyrosinase activity; and (*c*) we have been able to regulate independently the expression of tyrosinase and gp75 in melanoma cell lines (20).

Understanding the specificity of mAbs that react with melanosomal antigens will be important for sorting out the identity of these molecules. It has been proposed in a recent report by Jiménez et al. (41) that a second gene only distantly related to BBTY-1 and Pmel 34 (33), mapping to the b (brown) locus in the mouse (42), codes for a gene product with tyrosinase activity (41). Thus, it is becoming increasingly evident that tyrosinase is a member of a family of related molecules that include distinct genes and alternative transcripts of these genes (32–34, 36, 41, 42).

Summary

A distinguishing characteristic of cells of the melanocyte lineage is the expression of the melanosomal enzyme tyrosinase that catalyzes the synthesis of the pigment melanin. A tyrosinase cDNA clone, designated BBTY-1, was isolated from a library constructed from the pigmented TA99$^+$/CF21$^+$ melanoma cell line SK-MEL-19. Expression of BBTY-1 in mouse L929 fibroblasts led to synthesis and expression of active tyrosinase, and, unexpectedly, to stable production of melanin. Melanin was synthesized and stored within membrane-bound vesicles in the cytoplasm of transfected fibroblasts. BBTY-1 detected a 2.4-kb mRNA transcript in nine of nine pigmented, tyrosinase-positive melanoma cell lines. Tyrosinase transcripts of the same size and abundance were detected in a subset (three of eight) of nonpigmented, tyrosinase-negative melanoma cell lines, suggesting that post-transcriptional events are important in regulating tyrosinase activity. Two melanocyte antigens, recognized by mAbs TA99 and CF21, that are specifically located within melanosomes and are coexpressed with tyrosinase activity, did not react with transfected mouse fibroblasts expressing human tyrosinase, supporting the conclusion that these antigenic determinants are distinct from the tyrosinase molecule coded for by BBTY-1.

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